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The NMR Solution Structure of Calcium-Saturated Skeletal Muscle Troponin C.

by

Carolyn Marie Slupsky ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Fall 1995

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ABSTRACT

Over the past decade, there have been enormous changes in nuclear magnetic resonance (NMR) spectroscopy. The incorporation of isotope labels into bacterially expressed proteins, and the development of heteronuclear multidimensional NMR techniques have made it possible to solve the structures of proteins up to 25 kD. This thesis represents a major application of NMR to muscle protein research as it presents the structure of calcium-saturated troponin C (TnC), a muscle regulatory protein with molecular weight 18 315, which has not previously been solved in its calcium-saturated state.

TnC undergoes a calcium-induced dimerization at neutral pH. It is shown that the mode of dimerization involves the N-terminal domain of one monomer interacting with the N-terminal domain of another monomer. Addition of the solvent trifluoroethanol (TFE) to a concentration of 15% v:v results in a 10 fold increase in the dimer dissociation constant of calcium-saturated TnC. TFE, at the concentrations used herein, acts to perturb the quaternary structure, without adversely affecting the secondary or tertiary structure of TnC, making TnC predominantly a monomer for spectroscopic studies.

The tertiary structure elements of the N-terminal domain of TnC are very similar to those obtained for calmodulin and NTnC (residues 1-90 of TnC), whereas the tertiary structure elements for the C-terminal domain are most similar to the x-ray crystal structure of TnC. The structure of calcium-saturated TnC reveals a dumbbell shaped molecule, and upon calcium binding to the low affinity sites, a hydrophobic pocket opens whereupon helices B and C move away from helices N, A and D. Comparison of the solution structure of calcium-saturated TnC with the x-ray crystal structure of half-

saturated TnC (where the N-terminal domain sites are apo) reveals differences in the phi/psi angles of residue Glu 41, and in the linker region between the two domains (residues 85 to 94). The spatial relationship between the two domains is ill-defined due to residues in the linker region between the N and C terminal domains of TnC which are flexible.

Acknowledgments

I would first like to thank my supervisor, Brian Sykes, for providing such a stimulating environment for learning the intricacies of NMR. I would like to thank him for his encouragement, guidance and wisdom, for his trust and belief in my abilities, and for all the scientific and non-scientific discussions. I am grateful for the friendship we have developed over the years and especially for the many fun rounds of golf.

I am indebted to Stéphane Gagné who has shared all the fun and difficulties associated with developing 3D NMR in the laboratory. I am grateful to him for bringing to my attention and helping me use the processing and peak-picking programs NMRPIPE and PIPP. These programs were kindly provided to us by Dr. Frank Delaglio and Dr. Dan Garrett of the National Institutes of Health in Bethesda, Maryland. I am especially grateful for Stéphane's encouragement and friendship, for the many stimulating discussions, and for freely making available various programs and scripts which have made my life much simpler.

I am grateful to Frank Sönnichsen, Key-Sun Kim, and David Wishart who have provided me with an abundance of useful advice about solving protein structures. I am indebted to Leigh Willard who has always taken the time to help me with computing problems and questions, as well as for her help with programming and helping me to learn C. I am grateful for the friendship, advice and especially for our Monday night get-togethers.

I am grateful to Robert Boyko for help with incorporating new equations into his crvfit program, and for helping me the many times that data was lost on tape. I would like to thank Linda Golden for performing the experiments with glutathione, and for being a friend and source of support over the years. I would also like to thank David Corson for providing me with some of the labeled protein which I used in my studies, and Patricia Campbell for helpful advice with the TnC/TnIp interaction studies. I am indebted to Gary Shaw for help with the SCIII studies, for collaboration with the TnC/TnIp interaction studies, and especially for his encouragement and the many stimulating discussions.

I am grateful to Paul Semchuk for teaching me and helping me with peptide synthesis, HPLC purification, and mass spectroscopy. I am indebted to Kim Oikawa for helping me with CD studies, and Les Hicks for helping me with ultracentrifugation studies. I am indebted to Fernando Reinach for providing me with the TnC vector. I am especially grateful for all the encouragement, advice, and help from Dr. Cyril Kay, and Dr. Bob Hodges.

I am thankful to all the other members and friends of the lab who have helped me in various ways, and made my experience here enjoyable: Larry Calhoun, Sue Smith, Krishna Rajarathnam, John Bagu, Gerry McQuaid, Tim Jellard, and Ryan McKay.

I would like to thank my husband, Jason Sylvestre, for his gentle love and support. I am indebted to him for all of his help with WORD 5.0, and for proof-reading parts of this thesis.

Finally, I would like to thank my family, and especially my parents John and Jeanette for their constant and unfailing love, support, and belief in me, for helping me to proof-read parts of this thesis, and teaching me how to laugh and enjoy life.

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ABBREVIATIONS:

Boc	*tert*-butyloxycarbonyl.
CaM	calmodulin.
CD	circular dichroism.
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate.
CSI	chemical shift index.
DEAE	diethyl-aminoethyl.
DQF-COSY	two-dimensional double-quantum filtered correlation spectroscopy.
DSS	2,2'-dimethyl-2-silapentane-5-sulfonate.
DTNB	5,5'-dithiobis(2-nitrobenzoic acid).
DTT	dithiothreitol.
EDTA	ethylenediaminetetraacetic acid.
EGTA	ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
FID	free induction decay.
HCACO	three dimensional $^{1}\mathrm{H}\alpha$-$^{13}\mathrm{C}\alpha$-$^{13}\mathrm{C}'$ correlation.
HCCH-COSY	three dimensional $^{1}\mathrm{H}$-$^{13}\mathrm{C}$-$^{13}\mathrm{C}$-$^{1}\mathrm{H}$ correlation.
HMQC	heteronuclear multiple-quantum coherence.
HNCA	three dimensional $^{1}\mathrm{H}$-$^{15}\mathrm{N}$-$^{13}\mathrm{C}\alpha$ correlation.
HNCO	three dimensional $^{1}\mathrm{H}$-$^{15}\mathrm{N}$-$^{13}\mathrm{C}'_{(i-1)}$ correlation.
HN(CO)CA	three dimensional $^{1}\mathrm{H}$-$^{15}\mathrm{N}$-$^{13}\mathrm{C}\alpha_{(i-1)}$ correlation.
HOBt	1-hydroxybenzotriazole.
HPLC	high performance liquid chromatography.
HSQC	heteronuclear single quantum correlation.
IPTG	isopropyl β-D-thiogalactopyranoside.
K_d	dissociation constant
MLCK	myosin light chain kinase
MOPS	4-morpholinepropanesulfonic acid.
MW	molecular weight.
NMR	nuclear magnetic resonance.
NOE	nuclear Overhauser effect.
NOESY	nuclear Overhauser enhancement spectroscopy.
NTnC	N-terminal domain of recombinant chicken skeletal troponin C (residues 1-90).
OD	optical density.

PAGE	polyacrylamide gel electrophoresis.
ppm	parts per million.
SCIII	Ac-(A101) (Y112) s-TnC (93-126) amide. Synthetic peptide calcium binding site III of turkey skeletal TnC.
SCIV	Ac-s-TnC (129-162) amide. Synthetic peptide calcium binding site IV of turkey skeletal TnC.
SDS	sodium dodecyl sulfate.
s-TnC	skeletal troponin C (in chapters V to VII, recombinant chicken skeletal TnC, and in chapter VIII, turkey skeletal TnC).
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol. Unless otherwise stated, TFE will refer to 15% v/v TFE.
TH2	proteolytically derived (thrombin) calcium binding site IV of rabbit skeletal TnC (121 - 159).
TnC	troponin C (In all chapters, except chapter VIII (turkey skeletal troponin C), TnC refers to calcium saturated recombinant chicken skeletal troponin C which has three residues different from turkey skeletal TnC: A1 vs P1, I130 vs T130, and D133 vs E133).
TnI	troponin I.
TnIp	Nα-acetyl TnI (104-115) amide.
TnT	troponin T.
TOCSY	total correlation spectroscopy.
TRIS	tris(hydroxymethyl)aminomethane
TRNOE	transferred nuclear Overhauser enhancement.
TR$_2$C	C-terminal domain (residues 92-162) of turkey s-TnC.
UV	ultraviolet.
2D	two-dimensional.
3D	three-dimensional.

Ala (A)	Alanine.
Arg (R)	Arginine.
Asn (N)	Asparagine.
Asp (D)	Aspartic Acid.
Cys (C)	Cysteine.
Glu (E)	Glutamic Acid.
Gln (Q)	Glutamine.

Gly (G)	Glycine.
His (H)	Histidine.
Ile (I)	Isoleucine.
Leu (L)	Leucine.
Lys (K)	Lysine.
Met (M)	Methionine.
Phe (F)	Phenylalanine.
Pro (P)	Proline.
Ser (S)	Serine.
Thr (T)	Threonine.
Tyr (Y)	Tyrosine.
Trp (W)	Tryptophan.
Val (V)	Valine.

Chapter I: *Introduction*

Muscle tissue is important in the vertebrate body for producing motion, pumping the heart and operating the viscera and blood vessels. Muscles may be classified into three categories: skeletal, cardiac and smooth. Skeletal and cardiac muscles are very similar in protein content, but have differences in their mechanisms of excitation-contraction coupling. Smooth muscles, however, are different from both cardiac and skeletal muscles, and since smooth muscles are more difficult to work with experimentally, they are not as well understood. This thesis is a study of the structure of the calcium-sensitive trigger for muscle contraction, troponin C, in its calcium saturated form. In this chapter, the components of skeletal muscle tissue will be described together with studies of these proteins using nuclear magnetic resonance spectroscopy. Emphasis will be placed on the role of troponin C in muscle contraction. Next, the mechanism of skeletal muscle contraction will be discussed followed by a brief description of how calcium binds to calcium binding proteins and a comparison of calcium binding proteins which are structurally related to troponin C. Finally, this chapter will conclude with a summary of the history of nuclear magnetic resonance and how it has become a useful tool for solving protein structures.

The remaining chapters in this thesis will be devoted to developments in nuclear magnetic resonance spectroscopy which I have implemented in order to accomplish the structure of troponin C (Chapter II), experimental methods used (Chapter III), how modification of cysteine 101 affects the structure of a peptide homodimer representing calcium binding site III (Chapter IV), the use of trifluoroethanol as a denaturant of the quaternary structure TnC adopts upon saturation with calcium (Chapter V), the structural characterization of troponin C (Chapters VI - VII), the interaction of troponin C with a peptide representing the inhibitory region of troponin I (Chapter VIII), and finally this thesis will tie all the information obtained together in a conclusion, and propose future studies on this model system to further aid in understanding the mechanism of muscle contraction (Chapter IX).

I.1 Muscle Proteins - NMR studies.

This section presents studies, using NMR as a spectroscopic tool, of muscle proteins. For a more complete description of the study of these proteins, the reader is referred to several excellent reviews cited at the end of this section.

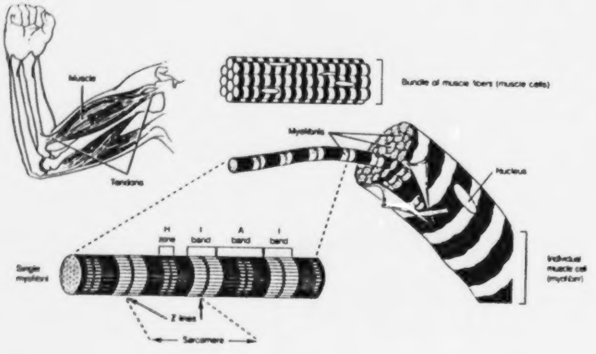
Muscle
Tendons
Bundle of muscle fibers (muscle cells)
Myofibrils
Nucleus
H zone
I band
A band
I band
Single myofibril
Individual muscle cell (myofiber)
Z lines
Sarcomere

Figure I.1. The levels of organization in striated muscle. Each muscle cell, or myofiber is multinucleate and contains many myofibrils. The myofibrils are in themselves repeating units, the functional unit being the sarcomere. Adapted from Matthews and vanHolde, 1990.

Skeletal muscles are composed of a large number of multi-nucleated cells (Figure I.1). A typical striated muscle contains bundles of muscle cells or muscle fibers. Each cell (which is referred to as a myofiber) contains myofibrils which are bundles of several hundred protein filaments in parallel. Under the microscope, the myofibrils exhibit a periodic structure of alternating dark (A) and light (I) bands. The dark bands in resting muscle have a lighter region termed the H-zone. The I bands are divided by thin lines called the Z-lines (Figure I.1). A sarcomere, then, is the repeating unit of the muscle taken from one Z-line to the next which is approximately 2.3 μm long in relaxed muscle (Matthews and vanHolde, 1990). Electron microscopy has shown that each sarcomere contains two types of filaments: thick filaments (which are composed of myosin and its associated light chains) and thin filaments (which are composed of actin, tropomyosin and the troponin complex, troponin I, troponin C, and troponin T). The thin filament extends in both directions from the Z-line and interdigitates with the thick filament in the

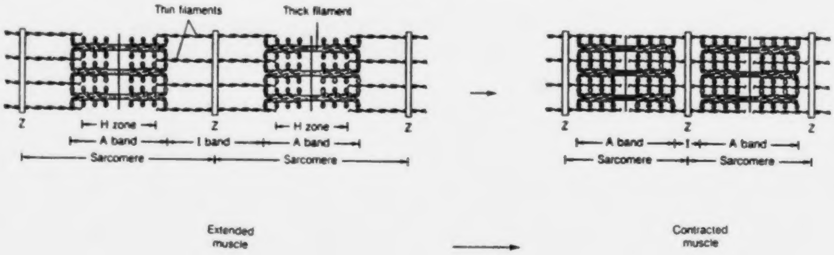
Thin filaments
Thick filament
Z
H zone
Z
H zone
Z
A band
I band
A band
Sarcomere
Sarcomere
Z
Z
Z
A band
I
A band
Sarcomere
Sarcomere
Extended muscle
Contracted muscle

Figure I.2. The sliding filament model of muscle contraction. Shown are two sarcomeres which are extended and contracted. Adapted from Matthews and vanHolde, 1990.

dark regions of the A band up to the edge of the H-zone (Figure I.2). In full contraction, each sarcomere shortens from 2.3 μm to 1.0 μm, and the I bands and H zones disappear moving the Z-lines up against the A band (Matthews and vanHolde, 1990).

I.1.1 Thick Filament proteins: *Myosin*

Myosin, the major component of the thick filament, is 300 to 500 nm in length (Darnell et al., 1990) and consists of two (220 kD) heavy chains which have associated four light chains that are between 15 and 22 kD in size (Rayment et al., 1993) (Figure I.3). Each heavy chain forms a globular head which contains an actin binding site and a nucleotide binding site (ATPase active site). Each chain also contains a long C-terminal α-helical tail which combines with another heavy chain to form the coiled-coil thick filament backbone. Myosin may be proteolytically cleaved by trypsin into light meromyosin (LMM) (the C-terminal α-helical coiled-coil portion of myosin), and heavy meromyosin (HMM) (the N-terminal portion of myosin containing the two globular heads and a portion of the coiled-coil structure) (Stryer, 1981). Further digestion of HMM by papain results in two globular heads with their associated light chains (S1) and the coiled-coil hinge region (S2) (Stryer, 1981).

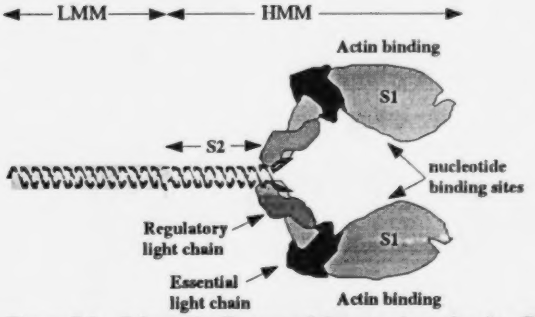

Figure I.3: Schematic diagram of the myosin molecule. Shown are the two heavy chains which form globular heads at their N-terminus (containing the actin binding site and the nucleotide binding site), and wind around one another at their C-terminus to form an α-helical coiled coil. The essential and regulatory light chains are as indicated. Myosin may be enzymatically cleaved with trypsin to form two cleavage products: LMM (light meromyosin) and HMM (heavy meromyosin) containing the globular heads, their associated light chains and a portion of the coiled-coil tail of myosin. HMM may be further cleaved by papain into S2 (coiled-coil hinge region) and S1 (Myosin globular heads with the associated light chains).

The crystal structure of myosin S1 has been determined to 2.8 Å resolution (Rayment et al., 1993) and is shown schematically in Figure I.3. S1 is approximately 45% α-helical. The nucleotide binding pocket is formed from several different α-helical and loop regions of the molecule. The actin binding site appears to be on the opposite side of the S1 head to the nucleotide binding site (Rayment et al., 1993). Associated with the globular S1 head are the essential and regulatory light chains. The essential light chain interacts with the C-terminal long helical end of S1 which presumably joins up to the α-helical coiled-coil backbone (Figure I.3). This light chain appears to have conformational flexibility in the crystal structure (Rayment et al., 1993). The regulatory light chain interacts with the region further to the C-terminal end of S1 (Rayment et al., 1993). Both the essential and regulatory light chains share sequence and structural homology to calmodulin and troponin C, although the essential light chain does not bind divalent cations, and the regulatory light chain can bind one divalent cation.

There have been many NMR studies of intact myosin (Riberio et al., 1984; Highsmith et al., 1979; Highsmith and Jardetzky, 1981; Eads and Mandeldern, 1984; Sommerville et al., 1990; Kalbitzer et al., 1992a), the light chains (Koppitz et al., 1980; Prince et al., 1981; Henry et al., 1982; Roux-Fromy and Cardinaud, 1984; Tollemar et al., 1986; Trayer et al., 1980; Bhandari et al., 1986; Trayer et al., 1987; Levine et al., 1988), the nucleotide binding site (Webb et al., 1978; Rösch et al., 1981; Shriver and Sykes, 1981a,b, 1982; Sykes, 1983; Baldo et al., 1983; Kay et al., 1987; Tanokura and Ebashi, 1993; André et al., 1989; Henry et al., 1993; Maruta et al., 1993), the actin binding site (Highsmith et al., 1979; Prince et al., 1981; Bhandari et al., 1986; Trayer et al., 1987; Dalgarno et al., 1982; Levine et al., 1991) the S2 region (Stewart and Roberts, 1982), and light meromyosin (LMM) (Kalbitzer et al., 1991).

NMR studies of myosin binding to actin have revealed two possible actin binding sites on myosin. The first site belongs to the essential light chain's N-terminal 41-residue segment (Bhandari et al., 1986; Trayer et al., 1987; Dalgarno et al., 1982) and the second site is close to the sulfhydryl group SH_1 on S1 (Barden and Phillips, 1990). Evidence for involvement of the essential light chain arises from a study in which actin was covalently labeled at cysteine 374 (near the C-terminus) with a spin label and the signal arising from the N-trimethyl protons of the essential light chain was monitored. This signal was broadened upon actin binding, indicating that these protons are within 15 Å of the C-terminus of actin (Trayer et al., 1987). The N-terminal region of the essential light chain was also able to alter the thermodynamic characteristics of the regulated acto-S1 interaction, thus operating as a functional linkage in the thin filament based regulatory mechanism (Bhandari et al., 1986).

S2 is the portion of myosin that connects S1 to LMM. It has been thought that this region of myosin contains a hinge which aids in the power stroke of myosin. Stewart and Roberts (1982) have used NMR to see if a flexible region associated with a hinge could be identified. Some sharp resonances could be identified (most likely due to side chain mobility) but not enough to suggest a major flexible region.

The essential light chain has two isoforms referred to as A1 (MW 25 kD) and A2 (MW 17 kD). These isoforms have an identical 141 residue C-terminus and are homologous over the preceding eight residues. A1 has a 41-residue N-terminal tail rich in proline, alanine and lysine residues. Isolation of residues 1 to 37 of A1 revealed that proline residues are primarily in the trans configuration, consistent with an elongated structure (Bhandari et al., 1986). Further, this conformation was found to be present in intact S1, suggesting that this segment may undergo lateral and rotational diffusion independent of the rest of the complex.

The regulatory light chain is thought to play a role in modulating the actomyosin interaction during long-term stimulation of muscle (Tollemar et al., 1986). The regulatory light chain was determined to bind a near stoichiometric amount of calcium with high affinity (Tollemar et al., 1986).

I.1.2. Thin Filament Proteins: *Actin*.

The thin filament is a complex of several proteins of which actin is the most abundant and the major structural component (Figure I.4). Actin interacts with tropomyosin and troponin in a calcium mediated manner to control muscle contraction. The interaction of actin with myosin mediates the force development in muscle.

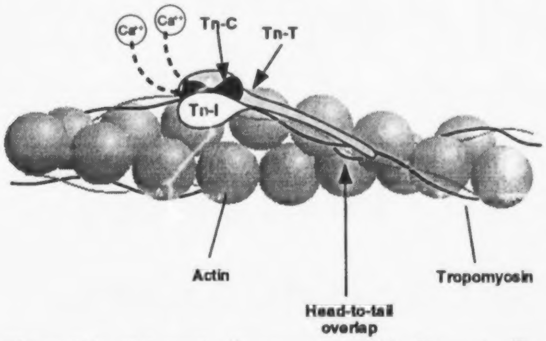

Figure I.4: Schematic diagram of the thin filament. The actin filament is composed of two strands of polymerized actin monomers. Tropomyosin, polymerized in a head to tail manner, lies near the grooves of the actin filament, one tropomyosin monomer spanning seven actin monomers. The troponin complex interacts with both actin and tropomyosin, the troponin T (TnT) component overlapping tropomyosin in its C-terminal region including the head-to-tail overlap. Troponin I (TnI) binds to TnC and actin and is the inhibitory component, and troponin C (TnC) is the calcium binding component responsible for the calcium sensitivity of the complex. This figure has been adapted from Heeley et al., 1987.

In the absence of salt, actin exists as a globular protein known as G-actin. G-actin contains one mol each of calcium or magnesium and ATP. G-actin changes upon the addition of salt into a highly asymmetrical fibrous polymer known as F-actin. Each actin monomer has 375 residues, five of which are sulfhydryl residues (residues 10, 217, 257, 285, and 374). The crystal structure of G-actin complexed with DNaseI has recently been determined to 2.8Å resolution with ATP bound and 3.0Å resolution with ADP bound (Kabsch et al., 1990) (Figure I.5). The actin structure has two domains with the nucleotide and calcium ion bound in the cleft between the two domains. The helical stacking of the monomers gives F-actin the appearance of two strands of beads wrapped around one another. Since the subunits are asymmetric, the F-actin fiber has a defined direction (Matthews and vanHolde, 1991).

The study of actin by NMR has involved labeling actin with ^{19}F reagents and observing actin polymerization or interaction of actin with other members of the contractile apparatus (Brauer and Sykes, 1986; Barden et al., 1989; Kalbitzer et al., 1992b; Barden and Phillips, 1990), studying the ^{31}P NMR spectra of bound ATP (Brauer and Sykes, 1981a,b; Cozzone et al., 1974), or studying synthetic peptides of actin (Sönnichsen et al., 1992; Van Eyk et al., 1991a). Through the use of NMR, it was determined that cysteine 374 and lysine 61 are near the polymerization site of actin. Further, lysine 61 is near where tropomyosin binds and where myosin S1 binds, supporting the view that myosin and tropomyosin compete for the same site on actin and that tropomyosin and myosin bind to F-actin in the groove formed by packing the actin monomers in the long pitch of helices (Barden and Phillips, 1990). It was also determined that ATP was in slow exchange on the NMR time scale and that it was constrained (Brauer and Sykes, 1981a, b; Cozzone et al., 1974). It was shown that the binding of calcium by G-actin was close to or involved the phosphate groups of ATP (Brauer and Sykes, 1981b, 1982; Kabsch et al., 1990). Upon exchange of magnesium for calcium, the conformation of actin changes. Concomitant with this, there is a six fold increase in the off rate of ATP, indicating that the conformational change, when magnesium binds, may be related to ATP hydrolysis (Brauer and Sykes, 1986).

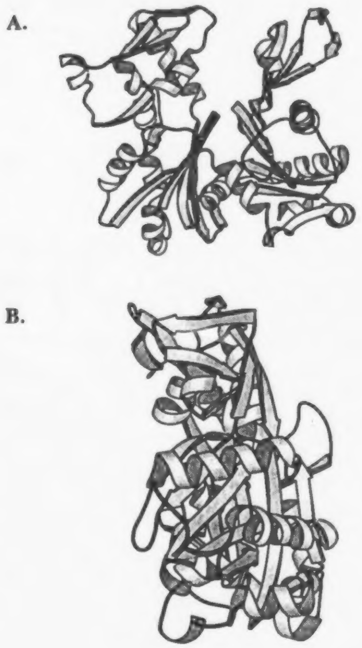

Figure I.5: A ribbon diagram of the 3D structure of actin. A. The molecule is presumed to have a similar orientation in the filament. The right side is thought to be exposed on the outer surface of the filament. The left side is thought to be near the axis of the filament. B. Side view of actin. This figure was prepared using MOLSCRIPT (Kraulis, 1991).

To study protein interaction, a synthetic peptide, encompassing residues 1-28 of actin, was shown to bind to S1 and increase the ATPase activity by 150% (Van Eyk et al., 1991a). Maximal activity occurred at a ratio of 0.5:1 peptide:HMM suggesting that one peptide binds to one head of the myosin dimer and cooperative interactions occur between the two heads. It was also shown that actin 1-28 binds troponin in the absence of calcium but not in its presence, indicating that the interaction between actin and TnI is sensitive to the calcium dependent changes in TnC (Van Eyk et al., 1991a). Further, a peptide of TnI, TnI 104 - 115, successfully competes with TnI for actin 1-28. Using the transferred NOE technique, the authors were able to determine that there is a change from an extended structure to a helix-like conformation between residues 1-7 of actin-1-28 when troponin is bound (Van Eyk et al., 1991a).

I.1.3. Thin Filament Proteins: *Tropomyosin*.

Tropomyosin is of central importance in the regulation of muscle contraction. Tropomyosin serves to propagate conformational changes which start in the troponin complex to the actomyosin ATPase. Tropomyosin is composed of two polypeptide chains, each 284 amino acid residues long and there are two species in skeletal muscle: α and β which share a high degree of similarity and the ratio of which varies depending on the type of muscle. Tropomyosin is an α-helical coiled-coil conformation except for the ends, the chains being parallel and in register. The ends of tropomyosin overlap with one another by eight to eleven residues in a head to tail fashion. Tropomyosin lies slightly out of the actin groove of the double helix in closer association with one actin chain rather than with the other, with one tropomyosin molecule spanning seven actin monomers (Figure I.4). Two sets of seven zones, each rotated 90° with respect to one another, bind to actin, and one tropomyosin molecule interacts with one troponin complex. It has been speculated that a change from the off state to the on state in muscle would involve one quarter turn of the tropomyosin molecule to the alternate set of sites (Leavis and Gergely, 1984). The troponin complex binds via the troponin T subunit to tropomyosin and extends over the entire C-terminal one-third of the tropomyosin molecule, including the region of the head-to-tail overlap, stabilizing the tropomyosin polymers (Leavis and Gergely, 1984).

There have been several NMR studies done involving tropomyosin mainly to describe the dynamics of the molecule (Edwards and Sykes, 1977, 1978, 1980, 1981; Sanders et al., 1988; Vogel and Bridger, 1983; Stewart and Roberts, 1983; Edwards et al., 1977; Brisson et al., 1986). The early ^{1}H NMR work involved observing tyrosine resonances and performing histidine titrations. This work revealed that tropomyosin is flexible at near neutral pH (Edwards and Sykes, 1977) and that two histidines which were separated by 180Å on the coiled-coil have significant cooperativity in their pH titrations only in polymerized tropomyosin. It was therefore concluded that the cooperativity must derive from a common conformational transition (Edwards and Sykes, 1981).

Troponin T increases the head to tail polymerization of tropomyosin. Work by Brisson et al., (1986) was able to show that CB1 (residues 1-151 of troponin T) binds close to the C-terminal end of tropomyosin by observing changes in the pH titration profile of histidine 153 of tropomyosin upon CB1 binding. This fragment of troponin T was able to invoke the same head-to-tail polymerization of tropomyosin as observed with whole TnT (Brisson et al., 1986). Further, CB1 was found to form a ternary complex with the N and C-terminal fragments of tropomyosin under conditions in which no interaction between the tropomyosin fragments could be detected (Brisson et al., 1986).

I.1.4. Thin Filament Proteins: *Troponin*

I.1.4.1 Troponin T (TnT)

TnT, as described above, has two main functions. These include anchoring the troponin complex to tropomyosin, and aiding in the head-to-tail polymerization of tropomyosin. TnT consists of one polypeptide chain with 259 amino acid residues and a molecular weight of 30 503 (Pearlstone and Smillie, 1976). TnT has approximately 50% polar residues and the charged residues are distributed more or less evenly throughout the sequence with the exception of the N-terminus (1-39), where acidic residues predominate, and the C-terminus (221-259), where there are predominantly basic residues. Hydrodynamic studies indicate that TnT is elongated rather than ellipsoidal (Prendergast and Potter, 1979). Electron micrographs show troponin to be globular with a rod-like tail portion, the globular part of the molecule containing TnC and TnI and the tail being TnT which is 185Å long and 20Å wide (Flicker et al., 1982). TnT binds directly to TnC, TnI, and tropomyosin (Leavis and Gergely, 1984), however the major interactions are to tropomyosin and TnI (Figure I.4).

Troponin T is the largest, and least well studied (by NMR methods) component of the troponin complex. TnT has two phosphorylation sites, one at the N-terminal serine hydroxyl, and one at serine 194 in the C-terminal cyanogen bromide fragment CN4 (Swiderek et al., 1990). The ^{31}P NMR spectrum of the N-terminal phosphorylated serine revealed a narrow resonance which titrated in pH identically to free phosphoserine, indicating that the phosphorylation site is highly mobile (Brauer and Sykes, 1984). The same result was obtained with CB1 (Brauer and Sykes, 1984) and when TnT was bound to the rest of the troponin complex or nonpolymerizable tropomyosin or tropomyosin fragments (Brauer and Sykes, 1984; Sperling et al., 1979).

I.1.4.2 Troponin I (TnI)

Troponin I is a protein of molecular weight 20 864 (Wilkinson and Grand, 1975) that inhibits the ATPase activity of actomyosin. The maximal inhibition by TnI in the absence of other regulatory proteins of the thin filament requires one TnI molecule per actin monomer, the interaction being independent of calcium. If tropomyosin is added to the system, one-half of the TnI molecules are needed for maximal inhibition suggesting that tropomyosin extends TnI inhibition over several actin monomers in the actin chain (Greaser and Gergely, 1973; Perry et al., 1972). The addition of TnC neutralized the inhibition of TnI in the presence and to a lesser extent in the absence of calcium (Weeks and Perry, 1978; Chong et al., 1983), but it was found that for fully reversible calcium inhibition, TnT was also required (Greaser and Gergely, 1973; Chong et al., 1983) (Figure I.4).

Labeling of lysine by reductive methylation or alkylation shows changes upon TnC binding in two areas: K31, K40 and K98, K105 and K107 (Lu et al., 1981; Hitchcock-Degregori, 1981). It was later found that the actual residues involved in the interaction were 1 - 21 and 96 - 116 of TnI. A CN4 peptide of TnI (96 - 116) was found to bind to actin and it was able to retain 40 to 75% of the inhibitory activity of TnI. The inhibition was enhanced by tropomyosin (Syska et al., 1976). The peptide 104 - 115 of TnI was found to be the minimum sequence necessary for the inhibition of the actomyosin ATPase activity. It has been shown that the interaction involving the N-terminal segment of the myosin essential light chain to actin is calcium sensitive, and the presence of TnI alone markedly inhibits the interaction (Grand et al., 1983).

Structural studies on whole TnI have not been accomplished so far due to the size of TnI (21 kD). In order to study the interaction of TnI and TnC, the synthetic peptide 105 - 115 (TnIp), which is the minimum sequence necessary for the inhibition of the actomyosin ATPase activity, was synthesized and bound to TnC, and the stoichiometry and structure of this fragment was determined (Campbell et al., 1991; Campbell and Sykes, 1991a,b). It was found that TnIp bound to TnC with a stoichiometry of binding of 1:1 (Campbell et al., 1991b). Using the transferred NOE experiment, the structure of the synthetic inhibitory peptide was determined when bound to TnC (Campbell and Sykes, 1991b). The structure revealed an amphiphilic α-helix distorted around the two central proline residues, bringing together the hydrophobic residues to form a hydrophobic pocket. The hydrophilic, basic residues extend from the opposite face of the peptide (Campbell et al., 1991; Campbell and Sykes, 1991b). The interaction of this peptide with TnC will be discussed in a later section.

I.1.4.3 Troponin C (TnC)

It was established in the mid-sixties that the calcium requirement for the ATPase activity of actomyosin depends on the presence of tropomyosin and troponin (Ebashi and Kodama, 1965, 1966). The first evidence that troponin consists of at least two proteins was suggested by Hartshorne and Mueller (1968) who were able to purify two fractions which they called troponin A and troponin B. Both fractions were required to confer calcium sensitivity on the actomyosin ATPase in the presence of tropomyosin (Hartshorne and Mueller, 1968). Troponin A, or EGTA sensitizing factor (Schaub and Perry, 1969), was later referred to as troponin C (TnC) since this was the protein responsible for the calcium sensitivity of the thin filament.

There are two types of TnC that may be purified either from fast twitch muscle or cardiac muscle. These two TnC isoforms have been highly conserved over millions of years of evolution with more than 90% identity between vertebrate species (comparing 10 sequenced TnC proteins of vertebrates from edible frog to human TnC using the program *seqsee*, (Wishart, Boyko, Willard and Sykes, unpublished results)).

I.1.4.3.1 Calcium Binding Sites

It was first suggested by Ebashi et al. (1968) that troponin has four calcium binding sites, two of which are high affinity and two of which are low affinity. Potter and Gergely (1975) and Potter et al. (1976) determined that the calcium binding component of troponin, TnC, has two high affinity sites which bind calcium and magnesium competitively ($K_{Ca} = 2.1 \times 10^7 \text{ M}^{-1}$ and $K_{Mg} = 5 \times 10^3 \text{ M}^{-1}$) and two low affinity sites which selectively bind calcium over magnesium ($K_{Ca} = 3 \times 10^5 \text{ M}^{-1}$). It was determined that in complexes of TnI/TnC and TnI/TnT/TnC, all four calcium binding sites have approximately the same affinity for calcium in the presence of 2 mM Mg^{2+}. Since the change in free magnesium concentration by approximately 2 mM did not affect calcium sensitivity of the myofibrillar ATPase activity, it was reasoned that the low affinity sites were the ones related to the regulation of muscle contraction (Potter and

Gergely, 1975). Analysis of the kinetics of calcium removal from TnC using stopped flow fluorimetric studies revealed that the release of calcium from the low affinity sites was much greater than the high affinity sites (Johnson et al., 1979). It was reasoned that since the events which regulate the skeletal muscle contraction-relaxation cycle must be complete within approximately 50 ms after excitation, only the calcium specific sites exchange rapidly enough to be involved in regulation. It was discovered that when a ternary complex of TnI, TnT and TnC is bound to the actin-tropomyosin complex, there is an increase in the Hill coefficient for the titration of the low affinity sites. This apparent cooperativity is further increased upon the addition of myosin (Grabarek et al., 1983). In the thin filament, however, it was shown that the calcium-specific sites have reduced calcium affinity (Zot et al., 1986).

There have been many studies of calcium binding using NMR. ^{1}H NMR studies were first done by Seamon et al (1977) and Levine et al (1977) and revealed that calcium induced a large folding of the backbone of the high affinity sites. Calcium binding to the low affinity sites did not alter the backbone of these sites significantly, but changed the hydrophobic interactions (Levine et al., 1977). A comparison of magnesium and calcium binding to the C-domain demonstrated differences in the degree of backbone folding of this domain and altered interactions between hydrophobic residues when magnesium was bound (Seamon et al., 1977; Levine et al., 1978), however magnesium was able to induce almost the same conformation as calcium (Seamon et al., 1977; Drakenberg et al., 1987). This led to the proposal that the important physiological sites were the N-terminal calcium specific sites and that the C-terminal sites were always filled with either calcium or magnesium. Tsuda and coworkers (1988, 1990), using high resolution ^{1}H NMR techniques, also studied magnesium binding to TnC. They demonstrated that all calcium binding sites in the N and C domains bound calcium and magnesium, but magnesium binding to the N-domain did not induce a conformational change in the hydrophobic region (Tsuda et al., 1990) since resonances in both domains were influenced by magnesium binding. Magnesium binding to the N-terminal domain of TnC was also demonstrated by Drakenberg et al. (1987). Studies of TnC from different sources such as rabbit and pike revealed very similar properties (McCubbin et al., 1982). Studies involving magnesium binding to acto-S1 ATPase have indicated that the high magnesium concentrations are important in the muscle cell as they promote muscle relaxation by binding to the C-terminal sites of TnC thereby causing troponin to be a more effective inhibitor of the actomyosin ATPase (Van Eyk et al., 1986).

Direct evidence for cooperative binding of calcium to the high affinity calcium binding sites was obtained using ^{113}Cd NMR (Teleman et al., 1983). Since ^{113}Cd has a spin I=1/2, a charge of +2, and an ionic radius which is similar to Ca^{2+}, it was the ideal choice for studying calcium binding. ^{1}H NMR proved that exactly the same spectral changes occurred upon binding of either Ca^{2+} or Cd^{2+} by TnC. A titration of TnC with Cd^{2+} revealed two ^{113}Cd NMR resonances which were equal in intensity irrespective of the amount of added Cd^{2+}. These results indicate that either the binding affinity is exactly the same for both sites or there is a strong positive cooperativity in binding between the two sites. A proteolytically derived fragment of the C-terminal domain of TnC had the identical ^{113}Cd spectra upon titration, indicating that the high affinity calcium binding sites were located in the C-terminal domain whereas the lower affinity calcium binding sites were located in the N-terminal domain (Drakenberg et al., 1987).

Calcium binding to the N-terminal calcium binding sites of TnC has previously been shown to be cooperative (Drakenberg et al., 1987; Golosinska et al., 1991; Pearlstone et al., 1992a,b; Li et al., 1994). Other studies have shown calcium binding to the N-terminal domain calcium binding sites to be non-cooperative (Grabarek et al., 1983). Two recent studies, one using bullfrog TnC (Imaizumi and Tanokura, 1990) and the other using the N-terminal domain (residues 1 - 90) of chicken recombinant TnC (Li et al., 1995) verify the latter result. Using microcalorimetry, Imaizumi and Tanokura (1990) were able to determine that calcium addition to the high affinity calcium binding sites of bullfrog TnC resulted in a sequestering of nonpolar groups to the interior of the molecule and a tightening of the molecular structure. The low affinity sites, however, were shown to bind sequentially, and that binding of calcium to the first low affinity site resulted in the exposure of hydrophobic groups from the interior of the molecule to the surface of the molecule. It was shown, using fluorescence spectroscopy, that calcium binding to either low affinity site produces the full fluorescence change (Zot and Potter, 1987a). Li et al., (1995) using the N-terminal domain of TnC and NMR as a spectroscopic tool, also demonstrated sequential binding of calcium to TnC. They were able to determine that the calcium dissociation constants were $0.8\ \mu M \leq K_{d1} \leq 3\ \mu M$, and $5\ \mu M \leq K_{d2} \leq 23\ \mu M$. Although, from these results, it is unclear which calcium binding site binds calcium first, it is plausible that loop II binds calcium first since in position Z (see Chapter I.2) of the calcium binding loop there is a serine (which interacts directly with Ca^{2+}) in site II and a glycine in site I (which presumably would need water to form a ligand to calcium). This replacement would most probably reduce the calcium affinity of site I relative to site II (Marsden et al., 1990). A study involving chimeric constructs of cardiac and skeletal isoforms of TnC seems to indicate that cardiac TnC site I is actively engaged in the trigger mechanism of TnC despite the inability to chelate calcium (Gulati et al., 1992).

There have been many studies involving whole TnC and synthetic peptides comprising the calcium binding loops of TnC in order to determine the calcium binding stability, and which residues are important in the calcium binding loop for maximal calcium affinity. These studies, as well as the structure of TnC, and studies on the calcium induced conformational change will be discussed in the next section on calcium binding proteins (Chapter I.2).

Calcium binding was shown to stabilize the two domains of TnC. A study of TnC using scanning microcalorimetry illustrated that TnC has two independent cooperative blocks, the stability of which depends on the calcium concentration (Tsalkova and Privalov, 1980). At saturating calcium concentrations the N-terminal domain is the most stable, followed by the C-terminal domain. It was also observed at saturating calcium concentrations that a third broad melting component appeared. The significance of this third block was not understood, however it will be shown in chapter V that it is most likely due to the melting of the calcium-induced dimer.

I.1.4.3.2 Interaction of TnC with other members of the troponin complex.

Over the years, it has become clear that the critical step in the regulatory process involves the interaction between TnC and TnI, and that the interaction between these two proteins is modulated by the presence or absence of calcium in the muscle. The binding constant for the formation of the TnC/TnI complex has been determined (Leavis et al., 1984; Ingraham and Swenson, 1984; Wang and Cheung, 1985; Cheung et al., 1987), and is somewhere between 10^6 M^{-1} in the presence of magnesium, and 10^9 M^{-1} in the presence of calcium. The cooperative strengthening of the linkage between TnI and TnC induced by calcium binding to the calcium specific sites of TnC, may have a direct relationship to the activation of the actomyosin ATPase (Wang and Cheung, 1985). Using fluorophores in reconstituted troponin, it was determined that regulatory calcium binding caused distance changes between the subunits in the troponin complex of approximately 0.7 to 1.1 nm (Schulzki, et al., 1990). While these distance changes are not large, the results suggest that there is indeed movement and restructuring of the troponin complex upon addition of calcium. Further, the fluorophoric sites selected may be localized in a zone of the troponin complex which is relatively little affected by the mechanism, thus accounting for the smaller than anticipated distance changes (Schulzki, et al., 1990).

Using CNBr fragments of TnI, two segments involved in the interaction of TnI with TnC were found: CN5 (residues 1 - 21), and CN4 (residues 96 - 116) (Moir et al., 1974; Cole and Perry, 1975; Syska et al., 1976). CN4 has been found to also bind to actin and to inhibit the actomyosin ATPase. It was determined that residues 104 to 115 of TnI comprise the minimum sequence necessary for the inhibition of actomyosin ATPase activity (Talbot and Hodges, 1979; Cachia et al., 1983, 1985; Van Eyk et al., 1991b). This peptide is extremely basic, with four arginines and two lysines within a 12 residue sequence. It has been determined that the most important residues for binding and inhibitory activity are F106, R108, L111, R113, V114, and R115 (Van Eyk and Hodges, 1988; Van Eyk et al., 1991b). The structure of the TnI peptide bound to calcium-saturated skeletal TnC was determined from 2D NOE ^{1}H NMR spectroscopy (Campbell and Sykes, 1989, 1991a,b). The structure suggests that residues 104 to 115 form an amphiphilic helix-like structure, distorted in the center by two proline residues when bound to TnC. The central bend in the peptide functions to bring the residues on the hydrophobic face into closer proximity with each other to form a small hydrophobic pocket with the hydrophilic basic residues extending off the opposite face of the peptide. Early ^{1}H-NMR work pointed to several positively charged and hydrophobic residues in the N-terminal portion of CN4 as residues involved in the interaction with TnC, whereas several Arg residues which are located mainly in the C-terminal portion of CN4 were involved in the interaction with actin (Dalgarno et al., 1982; Grand et al., 1982). Residues 6 - 16 of the CN5 segment of TnI have been shown to interact with TnC (Dalgarno et al., 1982). By attaching a paramagnetic spin label to cysteine 98 of rabbit skeletal TnC, it appeared that CN5 (residues 1 to 21) exhibited a U-shaped disposition about cysteine 98 of TnC, with the interaction being modulated by calcium binding to the low affinity calcium domain of TnC (Dalgarno et al., 1982). The reversal of the inhibition of the actomyosin ATPase by the inhibitory segment of TnI or TnI itself may be prevented by binding a synthetic peptide comprising residues 1 to 40 of TnI to TnC (Ngai and Hodges, 1992).

Recently it was determined, through the use of deletion mutants, that the C-terminal region of TnI, when linked to the inhibitory region, can regulate actomyosin ATPase (Farah et al., 1994). The C-terminal region itself, however, cannot. It was also determined that the N-terminal region or the N-terminal region with the inhibitory region of TnI cannot regulate actomyosin ATPase (Farah et al., 1994). Through binding studies, it was shown that the N-terminal region of TnI interacts with the C-terminal domain of TnC in the presence of calcium or magnesium and that the inhibitory plus the C-terminal region of TnI interacts with the N-terminal domain of TnC in a calcium dependent manner (Farah et al., 1994).

It was suggested from studies using TnC fragments that the helical regions (helices C, E, and G) located on the N-terminal sides of calcium-binding sites II, III, and IV interact with TnI (Grabarek et al., 1981; Leavis et al., 1978). TnC segment 89 to 100 of rabbit skeletal TnC is capable of releasing the inhibition of TnI, indicating that this region interacts with the inhibitory region of TnI (Weeks and Perry, 1978; Grabarek et al., 1981). Photochemical cross-linking of benzophenone attached to cysteine 98 of TnC readily occurs to the CN4 region of TnI (Leszyk et al., 1987, 1988), further supporting the suggestion that TnC (89-100) interacts with the inhibitory region of TnI. Using spin-labeled TnC, it was determined that the addition of calcium to binary complexes of spin-labeled TnC with TnT or TnI results in greater reduction in the mobility of the spin label versus the case of spin-labeled TnC alone (Ohnishi et al., 1975; Potter et al., 1976). Using zero length cross-linking (Leszyk et al., 1990), or a combination of site-directed mutagenesis and photoactivated cross-linking (Wang et al., 1990; Kobayashi et al., 1991), it was determined that the N-terminal domain of TnC (and in particular helix C, residue 57 of rabbit skeletal TnC) is close to the inhibitory region of TnI. It was also shown using ^{1}H NMR (Tsuda et al., 1992), and fluorescence (Swenson and Fredricksen, 1991) spectroscopies that the TnI inhibitory peptide can bind to fragments comprising the N or C terminal domains of TnC. See Chapter VIII for more discussion on the location of the inhibitory peptide on TnC.

It has been shown that the positively-charged side chains in the inhibitory segment of TnI are affected upon interaction with TnC (Grand et al., 1982), suggesting that hydrophobic as well as charge-charge interactions are involved in formation of the TnC-TnI complex. This has been suggested by Strynadka and James (1990) where, upon analysis of the crystal packing of $TnC\cdot 2Ca^{2+}$, it was observed that helix A of one TnC molecule interacts with the C-terminal domain of a symmetry related molecule. The hydrophobic contacts play a primary role in the binding of helix A, but some negatively charged groups surrounding the hydrophobic cleft also contribute to binding.

The topology of the interaction between TnI and TnC seems to point to both domains of TnC making contact with the inhibitory region of TnI, and the N and C terminal domains of TnC interacting with the opposite ends of the TnI inhibitory region (Kobayashi et al., 1994; Krudy et al., 1994). Using a combination of site-specific mutagenesis and photochemical crosslinking, it was determined that residue 12 of TnC crosslinked to TnI residues 132 - 141 at or near methionine 134, and residue 89 of TnC crosslinked to the inhibitory region of TnI (108 - 113) (Kobayashi et al., 1994). Using ^{13}C-methyl methionine labeled cardiac TnC, it was determined that the largest chemical shift changes occurred for methionines 81, 120, 137 and 157, 81 being located at the end of the N-terminal domain, and the other methionines being located in the C-terminal domain of TnC. Upon proteolysis to yield the N-terminal fragment 33 - 80 of TnI, only the C-terminal residues were observed to be perturbed (Krudy et al., 1994). These results are consistent with an antiparallel arrangement of TnC and TnI as was also shown by Farah et al. (1994). In binary or ternary complexes of TnI with other troponin subunits, cysteine 133 of TnI is exposed on the surface (Chong and Hodges, 1982a). Upon calcium binding to the N-terminal domain of TnC, it was shown that cysteine 133 of TnI moves toward cysteine 98 of TnC (Wang and Cheung, 1984; Tao et al., 1989), and away from actin (Tao et al., 1990) with only a minor alteration in the structure of TnI, since W158 and C133 of TnI do not move appreciably closer, but have a sharper distance distribution upon addition of calcium to the binary complex (Lakowicz et al., 1988). Further evidence for an antiparallel arrangement of TnC with TnI comes from cross-linking studies of Ngai et al. (1994) using TnIp (104 to 115) where it was observed that residue 104 of TnIp cross-linked to residue 155 of rabbit skeletal TnC. Iio (1993), observed that the C-terminal domain of melittin binds to the N-terminal domain of TnC.

Recent experiments using small angle x-ray and neutron scattering (Blechner et al., 1992; Olah et al., 1994) have suggested that the interconnecting helix of TnC is extended when TnC is complexed with TnI or a peptide of TnI (96 - 115). This work is consistent with that of Wang et al., (1987) and Kobayashi et al., (1994). A molecular modeling study (Olah and Trewhella, 1994) treated TnI as a helical spiral which winds through the hydrophobic regions of TnC. There are essentially three regions of TnI that interact with TnC: a central region that interacts with the length of TnC (the inhibitory region), and two end regions that interact primarily with the outer edges of the N- and C-terminal lobes of TnC (Olah and Trewhella, 1994). Although calmodulin (see Chapter I.2) contracts to bind myosin light chain kinase, it has also been observed to bind in an extended structure to subdomains of phosphorylase kinase (Trewhella et al., 1990), as was shown for TnC binding to TnI.

There have been many studies suggesting that TnC is more compact in solution (Heidorn and Trewhella, 1988; Hubbard et al., 1988; Cheung et al, 1991), and when in complex with TnI (Kobayashi et al., 1991; Cheung et al., 1991). There are many examples of other TnC-like proteins which adopt either a compact structure or an extended structure upon binding its target. The regulatory light chain of myosin is extended in the chicken skeletal myosin S1 fragment (Rayment et al., 1993) as well as in scallop myosin (Xie et al., 1994), with the helical region of the heavy chain twisting through the hydrophobic regions of the regulatory light chain in a manner similar to the results suggested for TnI in complex with $4Ca^{2+}$-TnC. The essential light chain binding to myosin, however, was shown to be similar to calmodulin bound to the MLCK peptides, but not as compact (Olah and Trewhella, 1994). TnC was shown to become more compact upon binding of melittin (Blechner et al., 1992), however mastoparan did not result in a contracted structure (Blechner et al., 1992) even though TnC binds one mastoparan molecule per TnC molecule, and there appears to be a mastoparan-induced interaction between the N and C terminal domains of TnC (Tsuda and Hikichi, 1992). It is thus reasonable to conclude that the interconnecting helix region is a point of potential flexibility for TnC or calmodulin in complex with various helical structures.

The interaction of TnC with TnT has also been studied. The C-terminal half (residues 84 to 135) of TnC crosslinks to residues 152 to 230 of TnT, with residues 175 to 178 being the most highly cross-linked region (Leszyk et al., 1988). The inhibitory region of TnI (96 to 116) was shown to bind to TnT (176 to 230). These results were also shown by Heeley et al., (1987) and photo-crosslinking studies by Tao et al. (1986) where cysteine 98 of TnC was shown to be in close contact with TnI and TnT, and all three subunits were shown to be near cysteine 190 of tropomyosin. Further, it was shown that residues 48 and 64 of TnI are within 14 Å of both TnI and TnT (Chong and Hodges, 1982b). Thus it appears that residues 28 to 82 of TnI interact with residues 135 to 185 of TnT.

In summary, the complex of TnI, TnC, and TnT appears to be as follows: The C-terminal domain of TnC (residues 87 to 162 of the chicken skeletal sequence) is involved in interactions with the C-terminal portion of TnT and the N-terminal portion of TnI. The N-terminal portion of TnI also interacts with the C-terminal portion of TnT. These results further support the hypothesis that the C-terminal domain of TnC anchors this protein to the troponin complex. The inhibitory sequence of TnI appears to interact with both domains of TnC and possibly forces the C-terminal portion of TnI to interact with the N-terminal domain of TnC upon saturation of TnC with calcium. The interaction between the TnI inhibitory region and helix C of TnC would require the N-terminal domain of TnC to swivel approximately 180° about the linker region and thus a flexible linker between the two domains is implied for this interaction to occur.

For general reviews of TnC and its function, the reader is referred to Leavis and Gergely (1984), Ohtsuki et al. (1986), Zot and Potter (1987b), Parmacek and Leiden (1991), and Grabarek et al. (1992).

This thesis focuses on using the recombinant form of chicken skeletal TnC and thus all further references will be to this form of TnC. The amino acid sequence for recombinant chicken skeletal TnC is as follows:

```
1     6     11    16    21    26    31    36    41
ASMTD QQAEA RAFLS EEMIA EFKAA FDMFD ADGGG DISTK ELGTV

46    51    56    61    66    71    76    81    86
MRMLG QNPTK EELDA IIEEV DEDGS GTIDF EEFLV MMVRQ MKEDA

91    96    101   106   111   116   121   126   131
KGKSE EELAN CFRIF DKNAD GFIDI EELGE ILRAT GEHVI EEDIE

136   141   146   151   156   161
DLMKD SDKNN DGRID FDEFL KMMEG VQ
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This protein belongs to a multigene family of calcium binding proteins which includes calmodulin, parvalbumin, calbindin and others and will be discussed further in the next section on calcium binding proteins.

I.1.5 Contraction:

The sliding filament model of muscle contraction was proposed by two independent groups of investigators, one led by Hugh Huxley (Huxley, 1957), and the other by Andrew Huxley (Huxley, 1957). The energy for the process is derived from the hydrolysis of ATP by the myosin S1 heads. According to the model, myosin headpieces "walk" along the interdigitated actin filaments pulling them past, thereby shortening the sarcomere (Matthews and vanHolde, 1990). The process of contraction is depicted in Figure I.6. Hydrolysis of ATP by myosin S1 is done in the absence of actin which results in a high energy conformation of myosin (step I). In the absence of calcium (less than approximately 10^{-7} M), myosin cannot bind to actin due to the inhibitory effect of TnI. Electrical depolarization of the muscle cell signals the release of calcium from the sarcoplasmic reticulum store into the cytoplasm such that the intracytoplasmic calcium concentration rises from approximately 10^{-7} M to 10^{-5} M. Some of this calcium binds to TnC, causing a conformational change which has been in the past poorly understood. This conformational change is transmitted throughout the troponin complex and tropomyosin, ultimately releasing the inhibitory action of TnI. Myosin is then able to bind to actin in its high energy conformation (step II), release its products (step III) and return to a low energy conformation upon binding ATP whereby it releases from actin (step IV). The cyclical process of ATP hydrolysis by myosin, binding of myosin to actin, release of ADP and Pi from myosin, followed by release of myosin from actin upon ATP binding pulls the thin filament toward the center of the sarcomere (see Figure I.2).

This thesis will uncover the poorly understood conformational change of TnC. The results presented will be one of the first steps to understanding the link in the signal between TnC and the formation of the myosin-actin crossbridges, and will help in understanding the general mechanism of calcium activation in this class of calcium binding proteins.

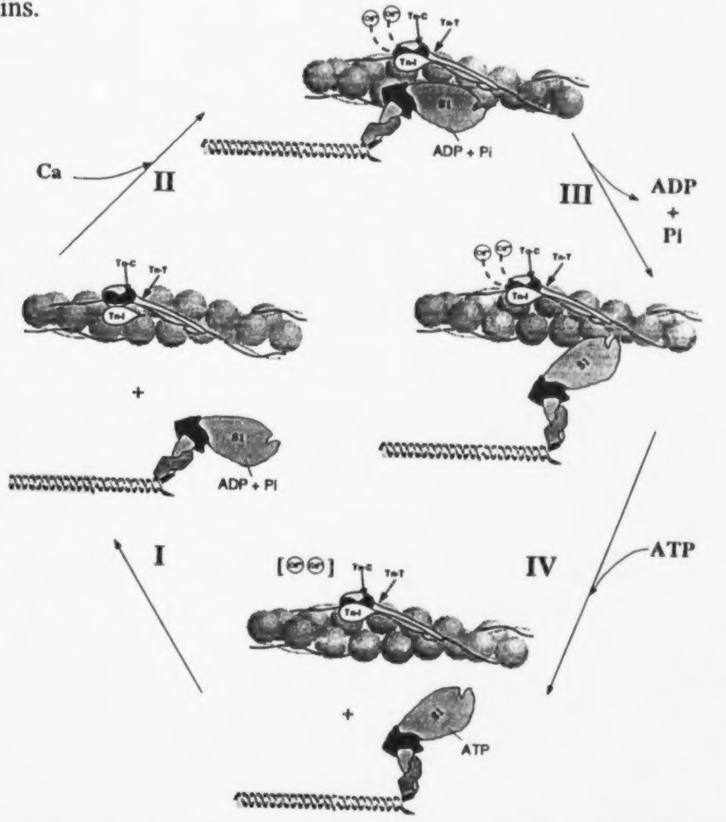

Figure I.6. Schematic diagram of the contraction process in skeletal muscle. The first step involves the hydrolysis of ATP to ADP and Pi. In the presence of calcium, myosin binds to actin, releases its products, and dissociates upon binding ATP to become ready for another contraction cycle. This cyclical attachment and detachment of myosin to actin is responsible for the myosin S1 heads "walking" along the actin filament.

I.2 Calcium Binding Proteins

Calcium is the fifth most abundant element on earth and is ubiquitous in biological organisms, processes and structures (McPhalen et al., 1991). Calcium is important for many processes such as muscle contraction and cell motility, nerve transmission, metabolic regulation, cell division and growth, secretion and membrane permeability (McPhalen et al., 1991). It is also essential for proper growth and maintenance of teeth, bones and shells.

Most calcium-dependent intracellular functions are regulated by calcium binding proteins. Calcium binding proteins comprise a large class of regulatory proteins which may be subdivided into two groups, each with distinctive structural features: the calcium-modulated proteins which utilize the EF-hand, and the annexin protein family which contain sequences resembling mutated EF-hands (Heizmann and Hunziker, 1991). The annexin protein family interacts with phospholipids and cellular membranes in a calcium dependent manner (Heizmann and Hunziker, 1991). This portion of Chapter I will describe those proteins in the calcium-modulated protein family (which include TnC, calmodulin, parvalbumin, calbindins, and other proteins) which are involved in regulation of cellular processes, and will describe structural aspects of these proteins in terms of their relationship to troponin C.

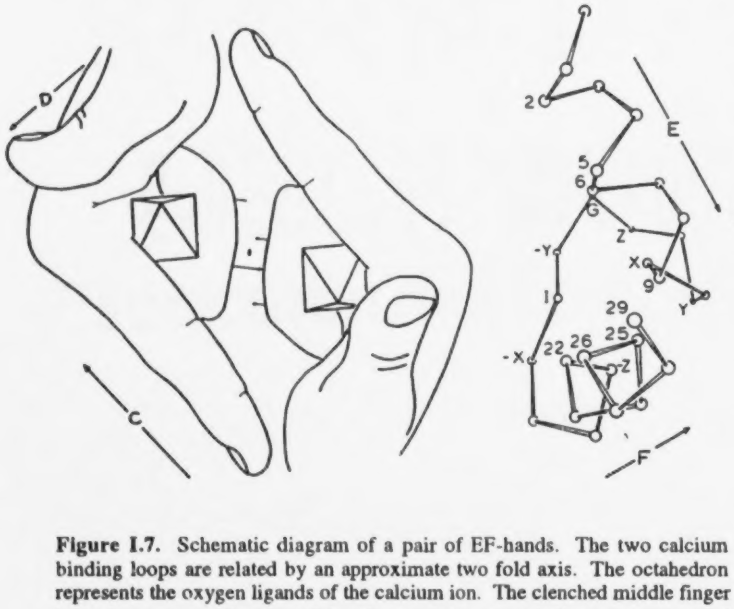

Figure I.7. Schematic diagram of a pair of EF-hands. The two calcium binding loops are related by an approximate two fold axis. The octahedron represents the oxygen ligands of the calcium ion. The clenched middle finger is the loop surrounding the calcium ion, and the two α-helices are represented by the extended forefinger and the thumb (from Kretsinger, 1980a).

I.2.1 How calcium is bound by calcium binding proteins.

The major feature of the calcium binding sites is a contiguous sequence of approximately 30 amino acids that form a helix-loop-helix (HLH) structural motif upon binding calcium (Kretsinger and Nockolds, 1973). These HLH calcium binding sites all begin with

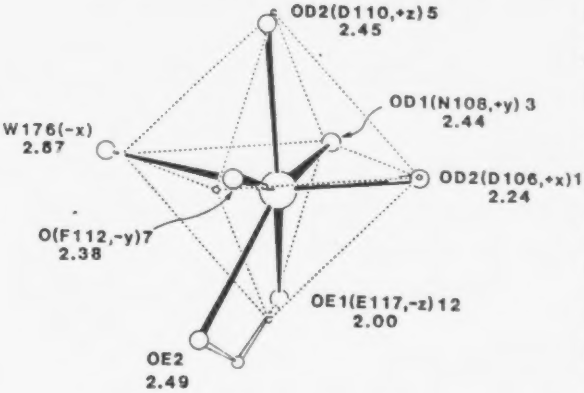

Figure I.8. A representation of the oxygen atom ligands of a calcium binding site illustrating the pentagonal bipyramidal geometry (adapted from Satyshur et al, 1988).

a γ turn followed by a three residue β-strand and end with the three amino-terminal residues of an α-helix (Strynadka and James, 1989). The HLH motif, or 'EF hand' as it is sometimes referred to, was first identified in the crystal structure of parvalbumin. It consists of two α-helices 'E' and 'F' joined by a calcium binding loop. The topology of the EF hand may be represented by a hand where the forefinger is helix E, the clenched middle finger represents the EF-loop, and helix F is the extended thumb of a right hand (Figure I.7). This EF hand is usually related by an approximate twofold axis of symmetry to another EF hand. Coordination of a metal ion occurs in a 12-residue loop region. Calcium or magnesium is ligated to the carboxyl, hydroxyl, or amide sidechains of five amino acid residues at positions 1 (X), 3 (Y), 5 (Z), 9 (-X), and 12 (-Z) and to the main chain carbonyl of the residue at position 7 (-Y). Seven oxygen ligands (the residue at position 12 is involved in a bidentate coordination with calcium) result in a pentagonal bipyramidal coordination geometry where each vertex is approximately 2.4 Å from the central calcium ion (Strynadka and James, 1989) (Figure I.8). Not all of the seven ligands are necessarily from the protein. There are cases where the calcium binding sites have one or more water molecules in the coordination sphere of the calcium ion (Strynadka and James, 1989).

It is well documented that the C-terminal domain of TnC is unstructured in the absence of calcium (Birnbaum and Sykes, 1978; Leavis et al., 1982; Drabikowski et al., 1985; Shaw et al., 1991a) and that the N-terminal domain appears to retain structure even in the absence of calcium (Leavis et al., 1982; Herzberg and James, 1988; Satyshur et al., 1988; Findlay et al., 1994; Findlay and Sykes, 1993; Gagné et al., 1994). In order to understand the structural aspects of calcium binding sites and to understand how calcium binds, studies involving synthetic peptides have been done. Upon synthesizing SCIII peptides (peptides representing calcium binding loop III of TnC) of differing helical lengths, it was demonstrated that the N-terminal and C-terminal helical regions stabilize the cation in the binding loop (Reid et al., 1981; Gariépy et al., 1982). It was also shown that interchain α-helical hydrophobic interactions contribute to the conformational stablilty of the SCIII peptide homodimer, as the alteration of leu 98, phe 102, ile 121 and leu 122 residues to alanine resulted in a 1000 fold decrease in calcium affinity with no alteration of the α-helix content (Monera et al., 1992). Further, the importance of metal charge and radius on the metal binding affinity has been studied (Reid and Hodges, 1980; Gariépy et al., 1985; Snyder et al., 1990). Other studies have involved determining the essential residues comprising the calcium sites (Kanellis et al., 1983; Boguta and Bierzynski, 1988; Marsden et al., 1990). Study of the calcium binding EF hands from many sources reveal that the invariant residues in the calcium binding loop are aspartic acid 1 (+x), aspartic acid/asparagine 3 (+y), glycine 6, isoleucine 8, and glutamic acid 12 (-z) (Marsden et al., 1990). Position 6 and 8 are highly conserved due to structural constraints. A glycine at position 6 allows ϕ, ψ space to be sampled in a region not allowed for a C_β containing amino acid $[(\phi,\psi) \approx (90^\circ, 0^\circ)]$, enabling the sidechain at position 5 and carbonyl main chain at position 7 to coordinate calcium. The residue at position 8 is involved in a β-sheet H-bonding interaction between adjacent calcium binding loops, which is needed for the construction of the hydrophobic core of the domain (Herzberg and James, 1985). It was shown that the helices surrounding the calcium binding loop account for 50% of the calcium binding affinity, while the interaction of the sites provide 43% (Marsden et al., 1990). A site-specific mutagenesis study of the calcium binding sites of skeletal TnC was able confirm the results of Marsden et al. (1990). In position x, mutation from an aspartic acid to a glutamine or asparagine residue yielded functionally inactive proteins with diminished calcium binding capacity. It was also shown for the -z position that neither aspartate nor glutamine could exchange for glutamate. For position y, asparagine or aspartate were functionally active, however glutamate was not (Babu et al., 1992). These mutations, however, may not necessarily alter the structural characteristics of the apo form of TnC since a mutant of cardiac TnC, where in site II the aspartic acid residue was mutated to an alanine residue to make the site functionally inactive, retained essentially the same structure as the apo form of the protein as determined using 2-dimensional NMR methods (Brito et al., 1991). A method has now been developed to predict the affinities of 'EF' hand pairs based on the net ligand charge of the two calcium binding loops, the hydrophobicity of the β-sheet segment of the loops and the hydrophobicity of the four 'EF-hand' helices (Sekharudu and Sundaralingam, 1988).

The first NMR structure of TnC fragments was determined by Shaw et al. (1990, 1992c). A 34 residue peptide corresponding to residues 93 to 126 of chicken skeletal troponin C was synthesized. This peptide was shown to be completely unstructured without calcium (Shaw et al., 1991a), but upon calcium binding formed a homodimer similar in tertiary structure to the C-terminal domain of TnC (as determined by x-ray crystallography). The dimer exhibited an approximate two fold rotational symmetry with hydrophobic residues and a three-residue β-sheet at the interface of the two sites, stabilizing the protein domain (Shaw et al., 1990, 1992c). This dimer was shown to bind calcium in a 1:2 calcium:peptide ratio with a $K_{d1} = 3$ μM, and a $K_{d2} > 1$ mM (Shaw et al, 1991a; see also Chapter IV). A similar dimer structure was found for a TH2 fragment (residues 121 - 159 of rabbit skeletal TnC) of TnC, although the calcium affinity for this calcium binding site is lower than for SCIII ($K_d = 1$ M) and a calcium:peptide ratio of 20:1 was required for dimer formation (Kay et al., 1991).

Mutation of SCIII calcium ligands to those of calcium binding site II of skeletal TnC decreased the K_d of the homodimer only slightly (Shaw et al., 1991b). Mutation of all residues in the calcium binding site to those of site II resulted in an even further decrease in K_d. The results suggest that differences in the coordinating ligands between calcium binding sites II and III have very little effect on calcium affinity. The non-coordinating ligands of site II, however, are responsible for the low affinity of site II as compared to the high affinity of site III (Shaw et al., 1991b).

When an equimolar mixture of 34-residue peptides representing SCIII and SCIV were mixed in the apo form, a ^{1}H NMR spectrum typical of a random coil conformation was observed. Upon addition of calcium, a heterodimer resulted with a calcium affinity greater than that of the homodimer (Shaw et al., 1991c, 1992a, 1992b). The calcium dissociation constants were determined to be $K_1 = 3$ μM and $K_2 = 2$ μM and the dissociation constant for the heterodimer was $K_d = 10$ μM (Shaw et al., 1992d). The ^{1}H NMR spectrum of the heterodimer was shown to be similar to the spectrum of TR2C (a proteolytic fragment of TnC representing residues 92 to 162 of chicken skeletal TnC) thus indicating that the heterodimer is a good model of the C-terminal domain of TnC.

Similar results have been shown for the intact cardiac TnC (cTnC) protein. Mutation of one of the residues in site III of cTnC, designed to prevent calcium binding to that site, resulted in a greater instability of the C-terminal domain versus a similar mutant in calcium binding site IV (Brito et al., 1993). This work suggests that calcium binding to site III was more crucial than calcium binding to site IV in stabilizing and folding the C-terminal domain of TnC.

In order to understand why a heterodimer is preferentially formed over homodimers upon mixture of SCIII and SCIV peptides, a study to determine the stability or free energy of formation of heterodimers and homodimers was undertaken (Shaw et al., 1994). It was shown that the SCIII/SCIV heterodimer is 3 kJ/mol more stable than the SCIII homodimer and 13 kJ/mol more stable than the SCIV homodimer (Shaw et al., 1994). Further, it was rationalized that since the free energy of folding for the SCIII/SCIV heterodimer (-65 kJ/mol) is significantly larger than for the homodimers (-58 kJ/mol), heterogenic two-site domains would be more commonly found than the homogeneous sites from which they most likely evolved (Shaw et al., 1994).

I.2.2. TnC

As previously mentioned, troponin C (TnC) is the calcium-binding regulatory protein of the troponin complex. Recombinant chicken skeletal TnC has a molecular weight of 18 257 and utilizes four EF-hand helix-loop-helix motifs for binding calcium. TnC is a very acidic protein with a pI of 4.1 to 4.4 (Hartshorne and Driezen, 1972). It was realized in the mid 1970's that TnC contained four homologous regions in the sequence (Collins et al., 1973; Collins, 1976; Kretsinger and Barry, 1975), each of which presumably contained one of the four calcium binding sites in a helix-loop-helix arrangement as described by Potter and Gergely (1975). Since then, there have been many studies on troponin C trying to delineate its calcium binding properties (see Chapter I.1), its structure and the conformational change that occurs upon binding calcium, the function of the long helical linker, and the interaction of this protein with other members of the troponin complex and in particular troponin I (see Chapter I.1).

I.2.2.1 X-ray crystallographic structure of $2Ca^{2+}\cdot$TnC.

The crystal structure of TnC with the high affinity calcium binding sites filled and the low affinity calcium binding sites unfilled has been solved to 2.0 Å resolution for turkey (Herzberg and James, 1988), and chicken (Satyshur et al., 1988) and more recently 1.78 Å resolution for chicken (Satyshur et al., 1994) skeletal TnC (Figure I.9). The structure reveals that TnC is a 70 Å long dumbbell-shaped molecule with approximately 66% of the structure being helical. The domains have a mean radii of approximately 17 Å, with their centers separated by a 31 residue α-helix corresponding to 44 Å (Herzberg and James, 1988). Each domain of TnC has two helix-loop-helix motifs tightly coupled by a short segment of β-sheet which helps to form the calcium binding loops. The N-terminal domain consists of helix A - calcium binding loop I - helix B and helix C - calcium binding loop II - helix D. The C-terminal domain consists of helix E - calcium binding loop III - helix F, and helix G - calcium binding loop IV - helix H. The N-terminal domain has an additional helix termed the N-helix that is apparently unique to the TnC's (Strynadka and James, 1989) and will be discussed in more detail (as to its function) in section I.2.2.3. In the crystal structure, the central connecting region is a continuous helix from helix D to helix E. Figure I.9 illustrates the major tertiary structural differences between the apo N-terminal domain and the 2-Ca^{2+} C-terminal domain of the crystal structure. On a superficial level, it appears that the N-terminal domain adopts a more closed structure whereas the C-terminal domain adopts a more open structure. An analysis of (ϕ,ψ) angles in the crystal structure reveals that one residue in helix B, E41, is irregular. This residue possesses irregular (ϕ,ψ) angles $[(\phi,\psi) = (-96^\circ, -7^\circ)]$ which produce a kink in helix B about residue 41. This irregularity appears for the modified structure of TR$_1$C (Findlay and Sykes, 1993; Findlay et al., 1994; Gagné et al., 1994) and the apo N-domain (residues 1 to 90) which will hereafter be referred to as NTnC (Gagné et al., 1994).

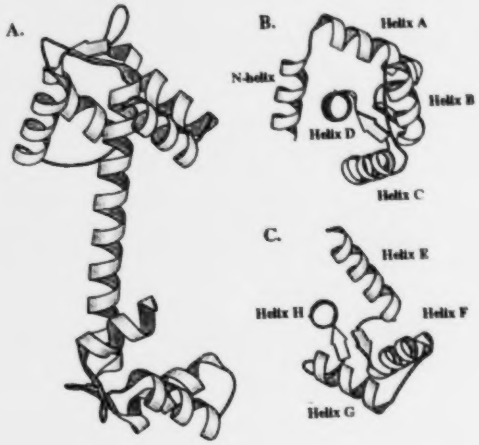

Figure I.9. x-ray crystal structure of TnC with the C-terminal domain calcium binding sites filled with calcium. A. The overall fold of TnC with two globular domains. B. The N-terminal domain overall fold. C. The C-terminal domain overall fold. This figure was prepared using MOLSCRIPT (Kraulis, 1991).

An analysis of interhelical angles for the C-terminal domain of the EF-hand of TnC reveals 113° for sites III and IV (Satyshur et al., 1988; Herzberg and James, 1988). In parvalbumin, these values are approximately 103 ° and in calbindin, 127°. For the N-terminal apo sites it was determined that the interhelical angles are 134° and 149° for the EF hand making them almost antiparallel versus perpendicular as for the C-terminal sites. From the crystal structure, it was determined that there are 72 hydrophobic contacts in the N-terminal domain versus 43 hydrophobic contacts in the C-terminal domain due to the larger number of hydrophobic residues in the N-terminal domain. It appears that the major difference between the N-terminal and C-terminal domains is the lack of hydrophobic contacts between the C-terminal domain F and H helices as compared to the many contacts between the B and D helices of the N-terminal domain. These differences were thought to play a role in the conformational transition and equilibrium between the metal bound and apo states (Herzberg and James, 1988; Satyshur et al., 1988).

I.2.2.2 The calcium-induced conformational change.

There have been many studies attempting to deduce exactly what the calcium-induced conformational change of TnC is. Circular dichroism measurements indicate that there is a conformational change upon calcium binding to the low affinity sites (Murray and Kay, 1972; Pearlstone, et al., 1992a,b; Li et al., 1994; Chandra et al., 1994). It was originally thought that an increase in α-helix occurs upon calcium addition, but as will be shown later, there is no change in the helical content of the N-terminal domain of TnC upon addition of calcium, but merely a rearrangement of helices. ^{1}H NMR studies on proteolytic fragments of TnC (Evans et al., 1980) have revealed that calcium binding to the low affinity sites results in a subtle alteration of the tertiary fold. This involved weakened contacts with several hydrophobic groups (Evans et al., 1980), and the phenylalanine resonances were observed to rearrange substantially upon binding of the third mol of calcium to cardiac TnC (Hincke et al., 1981; MacLachlan et al., 1990). Subtle alterations in the N-terminal domain (a tightening of the calcium binding loop and a strengthening of hydrogen bonds in β-strands) was seen for intact cardiac TnC (Krudy et al., 1992). Hydrophobic interaction chromatography has revealed that there is an opening of a hydrophobic patch in the N-terminal domain of TnC upon calcium binding (Vogel et al., 1983). This hydrophobic patch has a larger surface area than the C-terminal domain hydrophobic patch (since the C-terminal domain proteolytic fragment did not bind to the column). The surface area of the N-terminal domain hydrophobic patch was similar to the two halves of calmodulin since each of these bound to the hydrophobic column. Other studies have suggested that the TnC N-terminal domain swells upon addition of calcium (Fujisawa et al., 1990). It has also been shown that some residues go into a more hydrophobic environment upon addition of calcium (Johnson et al., 1978).

A model of the conformational change that occurs upon calcium binding to the N-terminal domain has been proposed by Herzberg et al. (1986). Using molecular modeling and assuming that the structure of the calcium bound form of the N-terminal domain should match the C-terminal domain, a model was formulated. Their model predicts that the major conformational transition upon binding calcium to the N-terminal domain is a movement of the B/C pair of helices away from the A/D pair. This movement would thus expose a patch of hydrophobic residues.

This model has support from several lines of evidence. It was shown that the reactivity of cysteine 84 in cardiac TnC increases upon calcium saturation of the N-terminal domain, supporting the model in that helix C separates from the central helix D, increasing the reactivity of cysteine 84 (Ingraham and Hodges, 1988; Fuchs et al., 1989). The formation of an intramolecular disulfide bond between cysteines 35 and 84 of cardiac TnC constrains TnC into an open conformation which can substitute for the calcium bound form of TnC in skeletal muscle (Putkey et al., 1993). Using ^{13}C-methyl labeled methionine and ^{13}C-^{1}H correlated NMR, cardiac TnC illustrated large chemical shift changes of methionine residues 45, 80, and 81 upon binding of calcium to the low affinity site II (Lin et al., 1994). These residues are located in helix B and helix D, further supporting the model of calcium saturated TnC. It should be pointed out that comparison of cardiac TnC and skeletal TnC reveals that the major difference between them is not merely that calcium can bind to site I in skeletal TnC and not in cardiac, but that the interaction of site I with the rest of the N-terminal domain determines the cardiac phenotype (Putkey et al., 1991; Gulati et al., 1992). The N-helix as well as the residues comprising site I are responsible for the cardiac phenotype (Gulati et al., 1992; Ding et al., 1994).

Site-directed mutagenesis was also able to confirm the model (for a mini-review, see daSilva and Reinach, 1991). Mutation of either glutamic acids 57 and 88 to a lysine reduced the calcium affinity of the N-terminal domain (Fujimori et al., 1990). It was reasoned that this would most likely be due to the formation of a salt-bridge which would stabilize the apo state. Mutation of Q48 and Q82 to cysteines, and cysteine 98 to leucine in rabbit skeletal TnC, and upon oxidation of the protein, calcium binding to the N-terminal domain was shown to be inhibited (Grabarek et al., 1990; Gusev et al., 1991). It was shown that the disulfide bridge had no effect on the secondary structure (Gusev et al., 1991), however interaction with TnI was inhibited. Double mutation of phenylalanine 29 to a tryptophan, and methionine 46 to a glutamine, or methionine 82 to a glutamine, or valine 45 to a threonine, resulted in an increase in the calcium affinity of the N-terminal domain validating the proposed model for the calcium-induced conformational change (Pearlstone et al., 1992a).

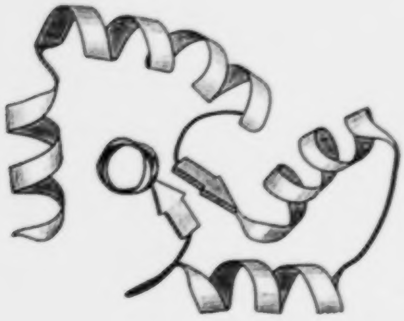

Figure I.10: Calcium-saturated NMR-determined structure of NTnC (residues 1 - 90). Coordinates for the average structure were kindly provided by S.M. Gagné. This figure was prepared using MOLSCRIPT (Kraulis, 1991)

Recently, the structures of NTnC apo and calcium saturated have been determined using 2D-^{1}H NMR spectroscopy as well as 3D heteronuclear NMR spectroscopy (Gagné et al., 1994, 1995) (Figure I.10). The structure of calcium saturated NTnC reveals an opening of the hydrophobic pocket of the N-terminal domain of NTnC by the altering of the (ϕ,ψ) angles of residue E41 in helix B from (-97°, -7°) to more helical values. This greatly alters the interhelical angles such that the structure is more open, with helices B and C moving away from helices N, A, and D. Upon comparison of this structure to the model, there are major differences in terms of interhelical angles. The NMR-determined structure reveals a more open structure than predicted by the model.

One potential problem with this structure determination is that at the concentrations used for the NMR experiment, TnC dimerizes. Further, TnC dimerizes when saturated with calcium via the N-domain of two monomers (see Chapter V). This thesis will address the structure of TnC as a monomer, as well as the structure of the entire molecule (versus one of the domains) (Chapters VI and VII). It will seek to identify the differences, if any, between the dimer and monomer structures, and if there are any major differences in structure between the whole molecule versus a particular domain.

I.2.2.3. The helical linker.

There have been a number of questions raised as to the stability of the apparent central helix proposed by the crystal structure of half saturated TnC. Consequently, there have been many studies to determine if this structure exists in solution or is merely an artifact of crystallization. Solution x-ray scattering studies have suggested that TnC is more compact at physiological pH and saturating calcium concentrations (Wang et al., 1987; Heidorn and Trewhella, 1988). At lower pH (pH 5.0), close to the pH at which crystals were obtained, it was demonstrated that TnC was elongated and similar to the crystal structure (Wang et al., 1987). The small-angle x-ray scattering studies of

Hubbard et al. (1988), however, suggest that the calcium bound form of TnC at pH 6.8 is only slightly more compact than the calcium free. One recent interesting study using monoclonal antibodies, however, would indicate that indeed skeletal TnC becomes more compact upon calcium saturation and increasing pH (Strang and Potter, 1992). Using fluorescence resonance energy transfer, it was shown that tyrosines in the N-terminal (position 10) and C-terminal (position 109) domains at least transiently come closer than 10 Å (Wang et al., 1993). This could only occur if there were present a flexible central helix in TnC which would result in either a spectrum of conformations or dynamic segmental flexibility (Wang et al., 1993).

There have been several recent studies using site-specific mutagenesis to delineate the function of the central helix. Mutation of glycine in the middle of the central helix to a proline, with the aim of disrupting the helix's integrity, had no effect on the activity of TnC (Reinach and Karlsson, 1988). By mutating calmodulin (deleting residues 81 - 84) and substituting it into muscle tissue for TnC, the force of the maximally activated fiber was found to be diminished (Gulati et al., 1990). Using deletion mutants of chicken skeletal TnC, it was determined that deletion of the tripeptides ^{91}KGK^{93}, or ^{88}EDA^{90} or the heptapeptide ^{87}KEDAKGK^{93} resulted in an altered interaction with troponin I in the absence of calcium (Dobrowolski et al., 1991). ^{88}EDA^{90} and ^{91}KG were shown to be unable to fully activate the actomyosin S1 ATPase in the presence of calcium. Thus, it was shown that changes in the central helix can alter the regulatory function of troponin C (Dobrowolski et al., 1991).

Another study in which the amino acid residues of the central helix were deleted, illustrated that up to seven amino acid residue deletions caused little change in the maximal force development in the muscle, but twelve deletions inhibited it (Babu et al., 1993). This study suggested that the critical role of the central helix is to keep the two lobes optimally apart, most likely in proximity of their respective target sites on troponin I in the fiber (Babu et al. 1993).

Perhaps the most intriguing study was by Gulati et al. (1993) where, upon deletion of the KGK triplet and removal of the N-helix, TnC elicited calmodulin-like regulation as tested by smooth muscle contractility and by the activation of phosphodiesterase. Further, replacement of the TnC residues ^{88}EDAKGK^{93} by the calmodulin specific DTD residues generated a highly effective calmodulin mimic irrespective of whether the N-helix was present or not. Since the calcium binding properties were unaffected, the difference had to do with the helical linker. It was suggested, therefore, that the TnC central helix evolved to be less pliable by the combined influences of ^{88}EDAKGK^{93} residues and the presence of the N-helix which together keep the N-terminal domain well separated from the C-terminal domain (Gulati et al., 1993). The recent low angle x-ray scattering studies would seem to support this (Olah et al., 1994).

It appears that the N-terminal helix of TnC is important for the function of TnC in the muscle (Smith et al., 1994; Chandra et al., 1994); however the affinity for the erythrocyte calcium-ATPase is only marginally affected by the deletion of the N-helix (daSilva et al., 1993). It was determined, however, that if glycine 92 is replaced with a leucine or alanine, the contractile defect associated with the N-helix deletion may by overcome (Ding et al., 1994). The results indicate that the destabilizing influence of glycine 92 in skeletal TnC is moderated by the N-helix.

1.2.3 Calmodulin

Calmodulin regulates a number of fundamental cellular activities such as cyclic nucleotide and glycogen metabolism, intracellular motility (microtubules and microfilaments), calcium transport and calcium-dependent protein kinases (Means and Dedman, 1980). It also mediates the activation of a number of different intracellular enzymes including phosphodiesterase, myosin light chain kinase (MLCK), calcineurin, erythrocyte calcium ATPase, brain adenylate cyclase, phosphorylase kinase and nicotinamide dinucleotide kinase (Strynadka and James, 1989). Calmodulin exists as a monomer of approximate molecular weight 17 000 and is very similar in tertiary structure to skeletal TnC. The major differences between calmodulin and TnC are the presence of an N-terminal helix in TnC of between 7 to 8 residues, 3 extra amino acid residues in the linker between the two domains (between 78 and 79 in calmodulin), and an extra residue at the C-terminus. There is approximately 45% direct homology between the two proteins and approximately 78% homology for conservative replacement upon alignment of the calcium binding loops.

As in the case with TnC, conformational changes produced by the binding of calcium are thought to result in the exposure of hydrophobic surfaces with which target enzymes or inhibitory drugs interact (Strynadka and James, 1989). The crystal structure has been refined to 2.2 Å (Babu et al., 1988) and 1.7 Å (Chattopadhyaya et al., 1992) in the presence of saturating calcium (both domains are in the calcium-bound form), and reveals an elongated protein that looks like a dumbbell (Figure I.11). Two domains are connected by a long α-helical linker, with each globular domain consisting of two EF hands in helix-loop helix conformations and a short stretch of antiparallel β-sheet between each pair of calcium-binding loops. Both domains of calmodulin are comparable in structure (RMSD = 0.751 Å) (Strynadka and James, 1989).

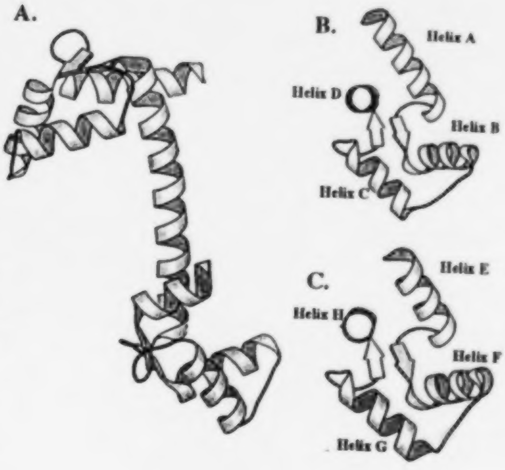

Figure I.11. A. Crystal structure of 4-Ca^{2+}-calmodulin as presented by Chattopadhyaya et al., (1992). B. N-terminal domain of calmodulin. C. C-terminal domain of calmodulin. This figure was prepared using MOLSCRIPT (Kraulis, 1991).

Since there is a three-residue difference in the helical linker, the orientation of the N- and C-terminal domains is different in the calmodulin crystal structure versus the crystal structure of TnC (the difference is approximately 60°, (Strynadka and James, 1989)). As with TnC, the structure of this helical linker has been challenged. X-ray solution scattering studies have indicated that the average distance between the two globular domains is several angstroms smaller in solution than in the crystal state (Heidorn and Trewhella, 1988). Solution NMR studies confirmed the x-ray scattering results (Ikura et al., 1991a; Barbato et al., 1992). Residues K77 to S81 exhibit a high degree of mobility and each domain tumbles independently of the other (Barbato et al., 1992). The rotational correlation times of each domain were determined to be τ_c ~ 6.3 ns and 7.1 ns for the C-terminal and N-terminal domains respectively.

The idea of a flexible tether between the two lobes of calmodulin was not new. Site-directed mutagenesis studies (Persechini and Kretsinger, 1988a) indicated flexibility. From these results, there have been several modeling studies on a bent calmodulin (Persechini and Kretsinger, 1988b; Vorherr et al., 1992; Strynadka and James, 1990; Mehler et al., 1991; Sekharudu and Sundaralingam, 1993). Upon complexation with the myosin light chain kinase peptide, it was determined that residues 75 to 77 change from α-helical to an extended conformation (Ikura et al., 1991a). This was also shown by a different group, however the extended residues reported from this group were 76 to 84 (Roth et al. 1992). It was also shown that the backbone conformation of the MLCK peptide was helical, confirming the amphiphilic helix model for the structure of peptides bound to calcium saturated calmodulin (Roth et al., 1991).

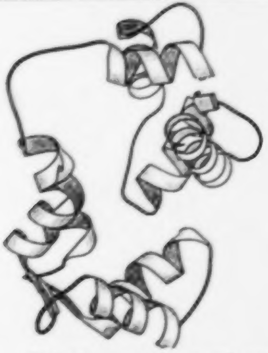

Figure I.12. Solution structure of calmodulin complexed with MLCK (Ikura et al., 1992). Shown is the structure of calmodulin only. This figure was prepared using MOLSCRIPT.

Structures have now been determined for a complex between calmodulin and a helical target peptide, MLCK, in solution (Ikura et al., 1992) and in the crystal state (Meador et al., 1992). It was found that the two domains (6 to 73, and 83 to 146) remain essentially unchanged upon complexation, with the central helix connecting the two domains (65 to 93) being disrupted into two helices connected by a flexible loop (74 to 82) (Ikura et al., 1992). This allows calmodulin to bind to residues 3 to 21 of the MLCK peptide which adopt a helical conformation. It was shown that the complex is stabilized by hydrophobic interactions including a large number of methionines (Ikura et al., 1992) (Figure I.12).

The three-dimensional structure of calmodulin, however, has only been solved and published in its calcium saturated form. Recently, a study of the apo form of the C-terminal domain of calmodulin was undertaken (Finn et al., 1993). It was found that the secondary structure was essentially the same as in the calcium activated form, however the secondary structure elements are rearranged so the hydrophobic binding pocket is closed in the apo form (Finn et al., 1993). A detailed structural study of the apo form of calmodulin suggests that the apo form of each domain is quite similar to the apo N-terminal domain of TnC (E31 and E104 have a kink in calmodulin creating a closed structure) (Ad Bax, personal communication). A full analysis and comparison will have to await the publication of this material in the near future.

1.2.4 Parvalbumin

Parvalbumin is a protein found in the cytosol of muscle cells and is thought to act as a one-way calcium messenger, being particularly important in cold-blooded animals such as fish and amphibians. Parvalbumins are essentially skeletal muscle proteins and are not found in cardiac or smooth muscle and therefore are not indispensable components of the contractile mechanism (Strynadka and James, 1989). Parvalbumins are also found in nervous tissue where synaptic transmission is triggered by calcium influx (Levine and Dalgarno, 1983). Parvalbumins are of low molecular weight (approximately 12 kD), and are characterized by a high calcium ion affinity (approximately 10^8 M^{-1}). Comparison of the amino acid sequences from different species indicated the presence of two distinct phylogenetic lineages termed α (pI 5.0), and β (pI 4.1). Parvalbumins of the β-series are characterized by a secondary cation binding site, which is inaccessible in parvalbumins of the α series (Cavé et al., 1982). The crystallographic structures of calcium saturated β-parvalbumin (Moews and Kretsinger, 1975) and α-parvalbumin (McPhalen et al., 1994) reveal globular molecules which are ellipsoid in shape (Figure I.13). Both α and β parvalbumins have essentially the same structure as can be seen in Figure I.13.

Parvalbumin contains six helices A through F, with helix C - loop region - helix D and helix E - loop region - helix F forming the two calcium binding loops. In addition, parvalbumin has a 39-residue N-terminal extension comprised of two helices which flank an eight-residue loop. These two helices and their loop region fold over the hydrophobic face formed by the two functional calcium binding loops, thus burying a central core of hydrophobic residues with an outer layer of hydrophilic residues. As a result, parvalbumin has none of the exposed hydrophobic surfaces that are thought to be the sites of target molecule binding as seen in TnC and Calmodulin (Strynadka and James, 1989). It is of interest to note that all calcium binding proteins give similar assigned NMR fingerprints for each pair of two hands, indicating that the EF-hands are similar between calcium binding proteins (Dalgarno et al., 1983a).

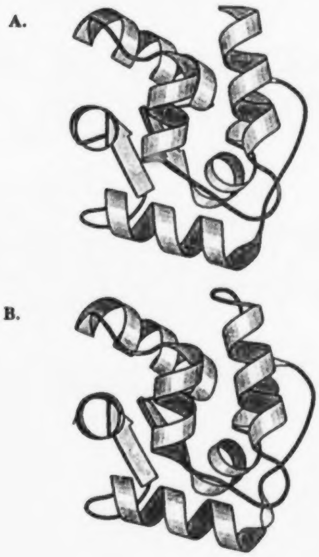

Figure I.13. Crystal structure of (A) calcium-saturated β-parvalbumin (Moews and Kretsinger, 1975), and (B) calcium-saturated α-parvalbumin (McPhalen et al., 1994). This figure was prepared using MOLSCRIPT (Kraulis, 1991).

NMR studies of parvalbumins are numerous. ^{1}H and ^{19}F NMR have revealed that upon calcium binding, there are structural rearrangements in the relative disposition of the helices resulting in the burying or exposure of residues (Opella et al., 1974; Bose and Bothner-By, 1983). Using 2D NMR techniques and distance geometry, the 3D structure of pike pI 5.0 parvalbumin (a member of the α-series) was determined (Padilla et al., 1988, 1989). It was shown to be similar to the crystal structure of β-

Parvalbumin and of course is similar to the structure of α-Parvalbumin. where helices A and B were present in an antiparallel arrangement, and C and D as well as E and F were present in a perpendicular arrangement with the CD and EF helices coming in close contact to form a short antiparallel β-sheet at the level of residues 57, 58, 59, and 96, 97, 98. α-Parvalbumin was shown to be similar to the crystal structure of the β-parvalbumins in the folding of its polypeptide chain, the difference being that α-parvalbumin has one extra residue at the C-terminal end of helix F (Padilla et al., 1988). The lengthening of this helix, it turns out, is a general feature of α-parvalbumins, resulting in an enhanced conformational stability due to this C-terminal region coming in close contact with helix B. Indeed, an NOE was observed between the C-terminal alanine 109 of helix F and lysine 27 of helix B (Padilla et al., 1988). It was also shown that the protonation / deprotonation of histidine 25 and histidine 106 causes a conformational change in the B-helix / F-helix region (Williams et al., 1986).

1.2.5 Calbindin

There are two types of calbindin; calbindin-D_{9K}, and calbindin-D_{28K}. These proteins bind calcium with high affinity ($K_a \sim 2 \times 10^6$) and are widely distributed in various species and organs. These proteins are hormonally controlled by vitamin D (hence the "D" in the name) and the number refers to the molecular weight. Although they are vitamin D dependent in tissues such as the intestine and kidney, there is evidence that they are not vitamin D dependent in tissues of the central nervous system (Gross and Kumar 1990). The highest concentrations of calbindin-D_{9K}, and calbindin-D_{28K} are found in calcium-transporting tissues such as the intestine, kidney and placenta, and smaller concentrations are found in nonepithelial tissues that do not transport calcium such as bone, parathyroid and brain (Gross and Kumar, 1990). Thus calbindin has a role mainly in calcium transport but may also be multifunctional.

Calbindin-D_{28K} is present in all vertebrate species and some invertebrates (for a review see Heizmann and Hunziker, 1990). It is thought that the protein may function as a mediator of vitamin D dependent calcium transport. This protein contains six putative calcium binding sites but only binds 3 to 4 mols with high affinity (Heizmann and Hunziker, 1990).

Calbindin D-9K has been studied more extensively. It is present mainly in mammalian intestine and appears to be involved in the translocation of calcium

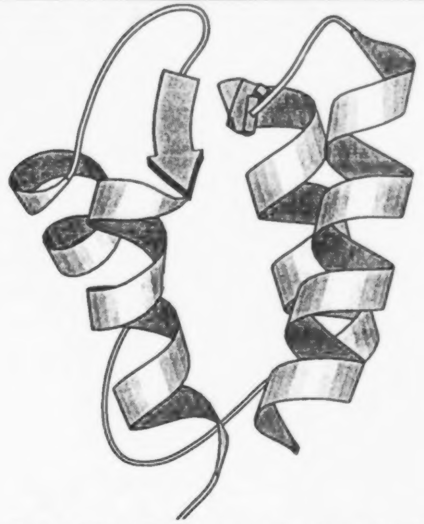

Figure I.14: The average structure of calcium-saturated calbindin-D9K as determined by NMR (Kördel et al., 1993). This figure was prepared using MOLSCRIPT (Kraulis, 1991).

(Heizmann and Hunziker, 1990). Calbindin-D_{9K} has two EF-hand calcium binding sites and binds two calcium ions strongly. The small size of calbindin-D_{9K} (75 residues) means it is ideal for study using NMR methods. Calbindin D_{9K} is comprised of four helices, two ion-binding loops, and a linker loop regardless of the calcium state. It has been determined that binding of calcium results in little change in the secondary structure of the protein, and that reorientation of sidechains occur at the helical interface formed by a pair of helix-loop-helix regions (Dalgarno et al., 1983b).

The three-dimensional calcium-saturated (Szebenyi and Moffat, 1986; Kördel et al., 1993) and apo (Skelton et al., 1994) calbindin-D_{9K} structures have been determined, and are very similar (Figure I.14). A comparison of the apo and $2Ca^{2+}$ structures reveals three primary effects associated with calcium binding: a change in the backbone conformation of helix IV from regular α-helix in the apo state to a mixture of 3_{10} and α-helix in the $2Ca^{2+}$ state, a rigid body movement of helix III of 1.5 Å with respect to the rest of the protein, and a reorganization of the packing of helices III and IV into each other and onto helices I and II (Skelton et al., 1994). It was reasoned that the asymmetry of the response to calcium binding arises because the side chains in the C-terminal EF-hand (helices III, and IV) need to be repositioned for chelation of the calcium ion, whereas the sidechain and backbone carbonyl groups are already well positioned for chelating the ion in the apo state (Skelton et al., 1994).

The difference between this protein and TnC or calmodulin is enormous. Although all bind calcium, it appears as if calbindin-D_{9K} does not have a significant rearrangement of helices in order to expose a hydrophobic pocket as do TnC or calmodulin. This difference in response is most likely due to the difference in function of the proteins. While TnC and calmodulin bind calcium and change conformation to interact with a target protein, calbindin's function appears to be merely as an intracellular buffer. As an intracellular buffer, calcium needs only to bind efficiently.

1.2.6 Other calcium binding proteins

As mentioned before there are two classes of light chains: essential and regulatory. Both classes of light chain subunits appear to have evolved from four-domain EF-hand proteins, but the essential light chains cannot bind calcium, and the regulatory light chains can bind only one calcium (Kretsinger et al., 1980b; Persechini et al., 1989). One study utilizing TnC/Regulatory light chain hybrids where calcium binding sites from TnC were spliced into the regulatory light chain protein, revealed that the hybrid proteins could bind to myosin and activate MgATPase activity, but they were unable to regulate it (daSilva et al., 1992). The structures of these proteins have been solved in complex with myosin (Rayment et al., 1993; Xie et al., 1994). In the structures, the regulatory light chain is extended with a kink in the interconnecting helix region so as to reorient the two globular lobes appropriately, whereas the essential light chain is similar to calmodulin bound to the MLCK peptides.

The S100 proteins are a group of dimeric proteins that are abundant in the brain, but are also found in chondrocytes, adipocytes, and slow-twitch muscle fibers. There are three forms (S100ao, S100a, and S100b) which have the subunit compositions of $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ respectively (Heizmann and Hunziker, 1991). The heterodimeric and homodimeric forms of S100 have different tissue distributions; the $\beta\beta$ dimer predominates in the brain (Persechini et al., 1989). S100 binds two calcium ions with lower affinity than some other calcium binding proteins (K_d = 60 and 200 μM) (Persechini et al., 1989). To date, very little has been accomplished on the S100 proteins in terms of structure elucidation.

Calcineurin is a type 2B protein phosphatase whose major substrates in brain appear to be the RII regulatory subunit of cAMP-dependent protein kinase and protein phosphatase 1 inhibitors, DARPP-32 and G protein (Persechini et al., 1989). Calcineurin has a broad specificity, causing phosphate groups to be removed from both protein and non-protein substrates (Heizmann and Hunziker, 1991). It has been identified as the intracellular target of the immunosuppressant-immunophilin complexes FKBP-FK506 and cyclophilin-cyclosporin A (Anglister et al., 1994). Calcineurin is a heterodimer consisting of subunit A (60 000 molecular weight), and subunit B (19 000 molecular weight). Subunit A has the catalytic and calmodulin-binding sites (Heizmann and Hunziker, 1991). Subunit B has approximately 35% sequence homology with calmodulin and contains four high-affinity calcium binding sites (Heizmann and Hunziker, 1991). The calcium binding loops share 54% sequence identity with the calcium binding loops of calmodulin, however outside these four loops, the degree of sequence homology is only 20% (Anglister et al., 1994). Recently, the secondary structure and NOE exchange rates were determined for calcineurin B (subunit B of calcineurin) using NMR spectroscopy (Anglister et al., 1993; Anglister et al., 1994; Grzesiek and Bax, 1993). It was determined, in the presence of CHAPS to prevent protein aggregation, that calcineurin B has eight helices distributed in four EF-hand calcium binding domains. The secondary structure appeared to be highly homologous to calmodulin (Anglister et al., 1994), except that helices B and C are shorter and helix G is longer in calcineurin versus calmodulin. It was also determined that the central helix is flexible (residues 84 to 88 which are analogous to 77 to 81 of calmodulin) (Grzesiek and Bax, 1993; Anglister et al., 1994).

Recoverin is a member of the EF-hand superfamily, and differs from calmodulin and troponin C in having additional residues at the amino and carboxy termini, plus an insertion between the third and fourth EF-hand domains. The recoverin family of proteins all appear to exhibit a calcium-myristoyl switch for membrane targeting not found in calmodulin or troponin C (Ames et al., 1994). Recoverin is a 23-kDa calcium-binding protein in retinal rod and cone cells, the calcium-bound form of which prolongs the photo-response most likely by blocking the phosphorylation of photoexcited rhodopsin (Ames et al., 1994). The crystal structure of unmyristoylated recoverin with one calcium ion bound has been solved (Flaherty et al., 1993), and reveals four EF-hand domains in a linear array. Calcium is bound to only one EF-hand (EF-3) in the crystal form. Recently, the study of myristoylated recoverin in the apo form using NMR has been undertaken (Ames et al., 1994). The secondary structure revealed 11 helical segments and two pairs of antiparallel β-sheets. It was determined that the N-terminal helix is longer and more flexible in this form of recoverin in contrast to what was obtained with the crystal structure of the unmyristoylated calcium-bound form.

For reviews of calcium binding proteins, the reader is referred to the following: Kretsinger, 1980b; Means and Dedman, 1980; Levine and Dalgarno, 1983; Persechini et al., 1989; Strynadka and James, 1989; Heizmann and Hunziker, 1990; McPhalen et al., 1991; and Heizmann and Hunziker, 1991.

I.3 Nuclear Magnetic Resonance Spectroscopy

The development of NMR spans many decades, beginning in the 1920's when the concepts of electron spin and the magnetic moment of the electron were formulated. Of particular importance was the Stern-Gerlach experiment where beams of atoms were separated in an inhomogeneous magnetic field according to the orientation of the electron magnetic moment (Becker, 1993). In 1939, a stream of hydrogen molecules was sent through both an inhomogeneous and a homogeneous magnetic field where the molecules were subjected to a radio-frequency electromagnetic energy (Kellogg et al., 1939; Rabi et al., 1939). The energy was absorbed by the molecules at a sharply defined frequency and this absorption was able to cause a small but measurable deflection of the beam. This truly was the first observation of the NMR experiment and for his accomplishments, Rabi received the Nobel prize in physics in 1944.

The next major development occurred independently by Bloch and Purcell in the 1940's and 50's. It was determined that by applying radiofrequency energy, the macroscopic nuclear magnetization could be rotated away from its equilibrium position parallel to the applied magnetic field. This displaced magnetization could then precess about the magnetic field at a certain energy. This precessing magnetization induced an electrical signal in an appropriately placed copper coil (Bloch et al., 1946; Purcell et al., 1946). For their independent experiments, both Bloch and Purcell received the Nobel prize in 1952.

It was in the 1950's that the concept of the chemical shift was founded. It was postulated that the magnetic properties of the electron surrounding the nucleus provides a shielding (σ) of the applied magnetic field, B_0. Thus, the nucleus will have a resonance frequency, $\omega = \gamma B_0(1-\sigma)$, where σ depends on the density and the configuration of the electrons. This shift in resonance frequency from what had been anticipated was called the chemical shift. This concept has provided the cornerstone for applying NMR to the solution of chemical and biochemical problems (Becker, 1993).

The ideas of spin-spin coupling and chemical exchange were also developed in the 1950's to account for multiple lines in a spectrum or lack thereof, and in the 1960's FT-NMR spectroscopy was developed in order to increase the signal to noise ratio (where N consecutive scans were recorded in digital memory and coherently added to produce a signal N times as large as one scan whereas random noise increased only by the square root of N times). The latter part of the 1960's and early 1970's saw the development of

NMR by Jeener (Becker, 1993). By the mid-70's, Richard Ernst started to develop the methodology of high resolution NMR spectroscopy. For his accomplishments, Ernst earned the Nobel prize in 1991.

This portion of chapter I will describe some basic concepts of NMR and their application to biological NMR. A complete description of NMR will not be given as it would exceed the size and scope of this thesis. For a more complete description of the theory of NMR, the reader is referred to several excellent books by Harris (1986), Ernst et al. (1987), Goldman (1991), Derome (1988), and Slichter (1963) as well as an excellent chapter in Allinger et al. (1976), and an excellent article by Kessler et al. (1988). These text-books are the source of information from which I have composed the following description for the general theory of NMR.

I.3.1 The NMR experiment: General Theory.

A proton has a spin quantum number of I = 1/2 as does the neutron. This spin quantum number reflects the fact that the nucleus has angular momentum or can spin. In general, nuclei with an odd mass number have half integral spin, nuclei with an even mass number and even charge number have zero spin, and nuclei with an even mass number but odd charge number have integral spin. Since NMR depends on the existence of nuclear spin, nuclei with I = 0 do not show any direct effects. ^{12}C, and ^{16}O isotopes account for most of the naturally occurring carbon and oxygen nuclei and therefore their effects may be ignored. ^{14}N has a spin quantum number of 1, but NMR absorption lines of I > 1/2 nuclei are generally broad due to the uncertainty principle. In this thesis, the nuclei that will be most commonly referred to are ^{1}H, ^{13}C, ^{15}N, and ^{19}F all with spin quantum number I = 1/2.

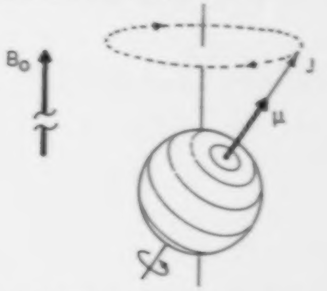

Figure I.15. A spinning charge with angular momentum J gives rise to a magnetic moment $\mu = \gamma J$. From Derome, 1988.

A spinning charge generates a magnetic field that has a magnetic moment μ associated with it. In the absence of a magnetic field each spin of an ensemble of spins will have the same nuclear spin energy, and each spin will be oriented in random directions. When an external magnetic field is applied (B_0), each nucleus will attempt to align its magnetic moment along the field direction (Figure I.15). Since only the component of angular momentum (J) can be altered by B_0, the net result corresponds to a rotation of the direction of μ in a cone with its axis along B_0. This movement is referred to as precession. The speed with which the magnetic moment rotates around the applied magnetic field is referred to as the Larmor frequency of the nucleus and is its NMR absorption frequency. This frequency (ω_0) depends on the strength of the applied field and on the intrinsic properties of the nucleus reflected in its gyromagnetic ratio γ. The Larmor frequency may therefore be represented by $\omega_0 = -\gamma B_0$. This rotation may be clockwise or counterclockwise depending on the sign of the gyromagnetic ratio, but is the same for any particular nucleus.

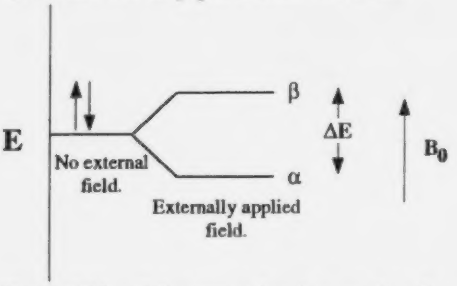

Figure I.16. Spin energy-level separation for a hydrogen nucleus as a function of B_0.

Each magnetic nucleus has 2I + 1 possible orientations and corresponding energy levels with respect to the external magnetic field. Thus, for the nuclei aforementioned (namely ^{1}H, ^{13}C, ^{15}N, or ^{19}F), all with I = 1/2, there are two possible orientations in the magnetic field: parallel and antiparallel. The energy difference between these two orientations is directly proportional to the strength of the magnetic field $\Delta E = \gamma h B_0 / 2\pi$ where h refers to Planck's constant (Figure I.16). At any given field strength, a nucleus can go from one energy level to the other by absorbing or emitting a discrete amount of energy at its Larmor frequency. Thus, it can be seen that when protons are placed in a magnetic field of approximately 141 000 Gauss, they precess at approximately 600 MHz. The absorption of energy at a particular frequency is referred to as resonance.

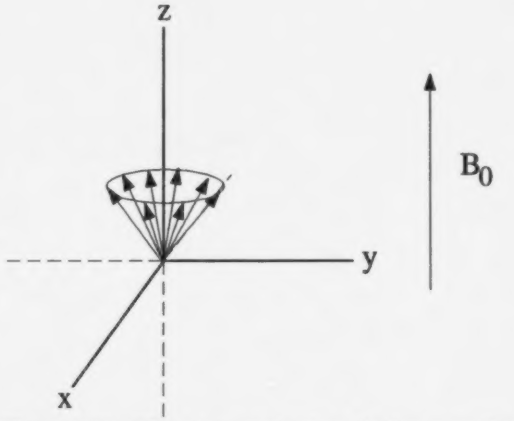

Figure I.17. With a large collection of spins, a surplus will have their z-components aligned with the applied field thereby becoming magnetized in that direction. Adapted from Derome, 1988.

Defining the z-axis to point along the static field direction, one spin will have a stationary component of its magnetic moment aligned along z and a component rotating with Larmor frequency in the x-y plane. If we consider a large number of spins, all with the same Larmor frequency (Figure I.17), the parallel orientation of the z-component of each spin is of lower energy than the antiparallel component. If thermal equilibrium is achieved, then differences in the population of two states will exist (more in the lower energy state according to the Boltzmann distribution). Thus, the net magnetization will align parallel with the magnetic field. Since the phase of the precession is random, there will be no net magnetization in the x-y plane for a large number of spins and therefore the total magnetization of the sample will be stationary along the z-axis.

All future discussions of ensembles of spins will take place in the rotating frame, that is, a set of coordinates will be chosen that rotates along with the nuclear precession at the same speed and direction. Therefore, each nuclear magnetic moment should appear static.

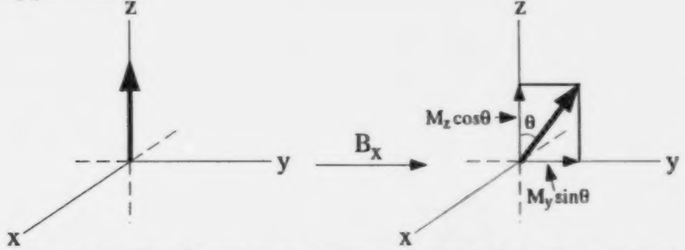

Figure I.18. The effect of an on-resonance rf pulse in the x-direction in the rotating frame and a flip angle θ on longitudinal magnetization.

Any particular NMR experiment is a collection of pulses and delays. Pulses may be quite simply thought of as the application of a radiofrequency (rf) field B_1 which is perpendicular to the static magnetic field (B_0). Pulses are short term high-frequency oscillating magnetic fields of the form $B_1\cos\omega_0 t$. Since two magnetizations at right angles exert a force on one another, the net result is that the sample magnetization is driven around the B_1 field vector at a speed depending on the field strength (Figure I.18). Another way to put this is that the magnetization rotates by a certain angle, the so-called flip angle, depending on the duration of the B_1 field (pulse length). Therefore pulses are usually characterized by a flip angle rather than a field strength or duration. A 90° pulse is therefore the time it takes a particular field strength to rotate the magnetization 90° or π/2 radians with respect to the z-axis. Removal of the rf field means the sample magnetization will attempt to precess about the z-axis at its Larmor frequency to reach its equilibrium position. This rotating vector will have components of x and y magnetization which are orthogonal. The signals measured along the x and y planes are thus taken separately or combined to form the Free Induction Decay (FID) which dies to zero as the magnetization reaches its equilibrium value (Figure I.19). Since B_1 rf fields cover a range of frequencies, the result is a FID corresponding to several frequencies at once. A mathematical operation called a Fourier Transform (FT) transforms frequencies, which are detected as oscillating functions of time, into signals which correspond to positions on a frequency scale.

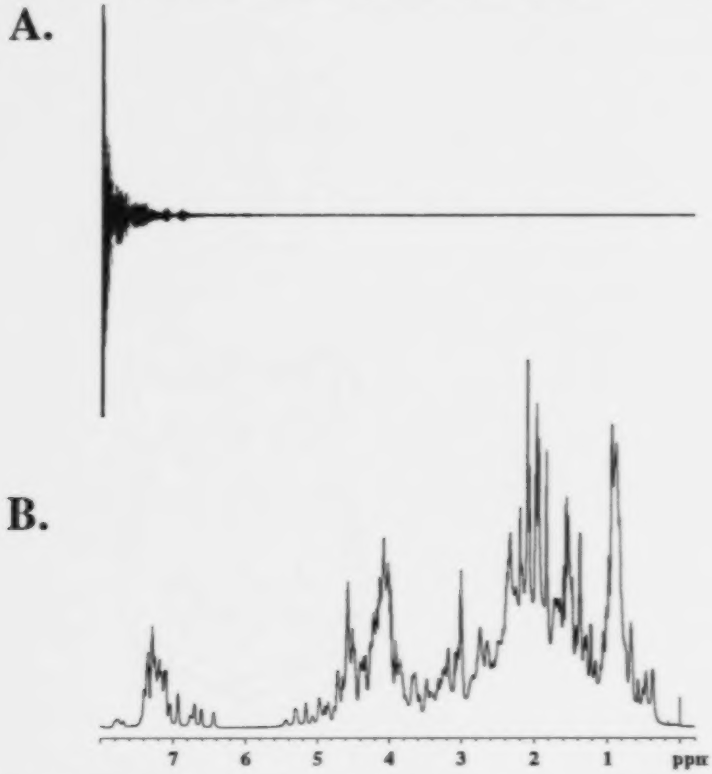

Figure I.19. A FID (A.) and its corresponding spectrum (B). Illustrated is a 1-D spectrum of TnC.

I.3.2. The Chemical Shift.

The field strength required for energy absorption by a given nucleus depends upon its environment. The field strength at which a particular nucleus absorbs will give information about the local molecular structure. For example, if we consider a hydrogen atom in a molecule, the magnetic field will induce a circulation of electrons around the hydrogen atom in a plane perpendicular to the external magnetic field. This circulating charge will generate an induced magnetic field around the hydrogen atom. Shielding of the hydrogen atom occurs when the electrons surrounding it induce a field that opposes the external field. Conversely, deshielding occurs when the induced field augments the external field. Because the shielding depends on the chemical environments, the field strengths required for energy absorption by different atoms are referred to as chemically shifted relative to a standard. DSS (2,2'-dimethyl-2-silapentane-5-sulfonate) is the standard used in these studies for ^{1}H and ^{13}C chemical shifts, NH_4Cl is the standard for

^{15}N chemical shifts, and TFA (trifluoroacetic acid) is the standard for ^{19}F chemical shifts. Chemical shift, then, is the difference between the field strengths at which the particular nucleus absorbs and the nuclei of the standard absorb. The δ scale is most commonly used as a measure of reporting chemical shifts.

$$\delta = \frac{\text{Shift from standard (Hz) x } 10^6}{\text{Spectrometer Frequency (Hz)}}(ppm)$$

I.3.3. Scalar Coupling.

Scalar coupling is essentially the interaction between two spins which occurs predominantly through bonds. It leads to splitting of resonances in an NMR spectrum. Another way to state this phenomenon is that the effect of the spin of one nucleus is transferred to the adjacent chemically different nucleus through the bonding electrons (the coupling is essentially between atoms one to three bonds apart). The adjacent nucleus then experiences a different effective magnetic field than in the absence of the original nucleus. The influence of the original nucleus on the strength of the net magnetic field experienced by the adjacent nucleus depends on the orientation of the spin of the adjacent nucleus relative to the external magnetic field (which can only be either parallel or antiparallel).

For example, let us consider two spins (spin 1 and spin 2). If spin 1 is undergoing resonance, it will feel both the applied field and the field from spin 2. Since spin 2 can be either parallel or antiparallel with respect to the external field, the field generated by spin 2 can either augment or decrement the field felt by spin 1. This augmentation or decrementation will cause the frequency of spin 1 to split and the resonance will appear as a doublet in the NMR spectrum. Since the energy difference between the two states is small, almost equal numbers of nuclei will occur in each state and therefore the components will have equal intensity.

The separation between the components of the doublet is called the coupling constant, J which is expressed in Hz. The oldest 2D NMR pulse scheme, in which the magnetization is transferred from one proton to another via ^1H - ^1H J coupling is called the COSY experiment. This experiment requires that the J_{HH} be not much smaller than the ^1H resonance linewidth. Since this linewidth is proportional to the inverse of the tumbling rate, it increases linearly with the size of the protein (Bax and Grzesiek, 1993).

Other J-correlation techniques correlate the frequency of a proton with that of its directly attached heteroatom (^{13}C or ^{15}N). The heteronuclear one-bond couplings, $^{1}J_{CH}$ (approximately 125 to 160 Hz) and $^{1}J_{NH}$ (approximately 95 Hz), are much larger than the $^{3}J_{HH}$ couplings (typically 4 to 12 Hz). Frequently as much as 50 to 90% of the magnetization can be transferred from protons to the directly coupled heteronuclei, and therefore the 2D heteronuclear shift correlation experiments are highly sensitive (Bax and Grzesiek, 1993). With isotopic enrichment of ^{13}C and ^{15}N into proteins, these types of experiments may be used for higher-dimensional NMR as will be discussed later (Figure I.20).

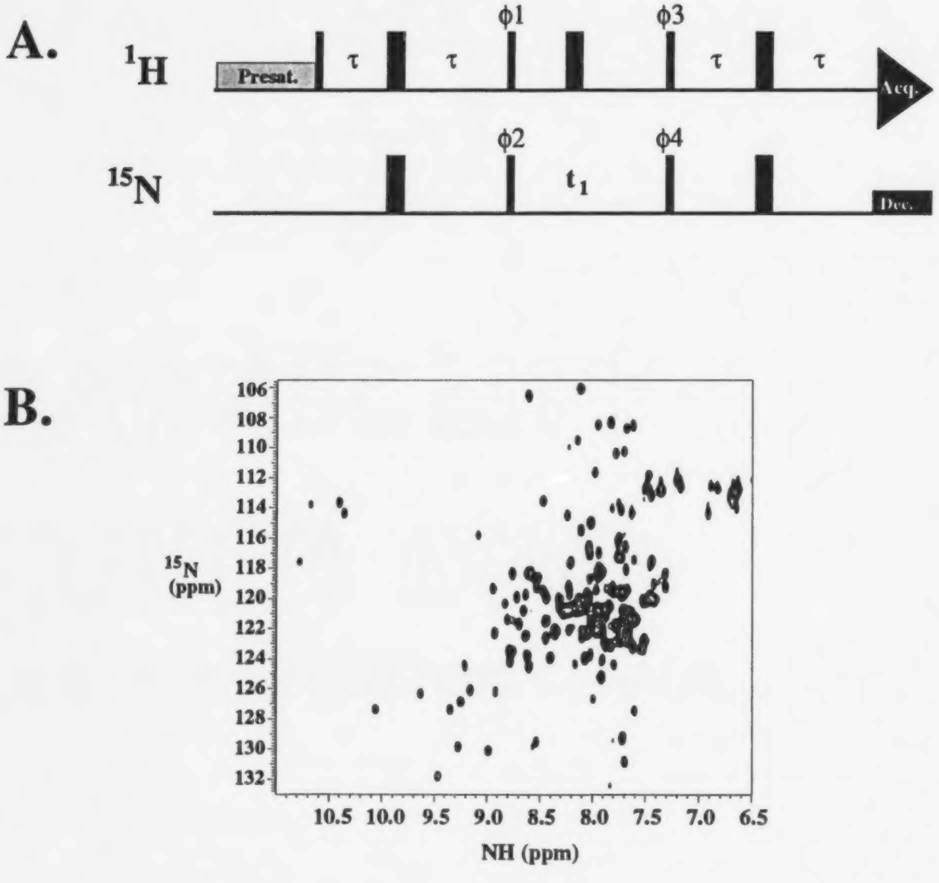

Figure I.20. (A) Pulse scheme for a heteronuclear single quantum shift correlation experiment (HSQC) in which ^{1}H's are coupled to their attached ^{15}N nuclei. (B) A typical HSQC spectrum of TnC.

Besides using J-correlated spectroscopy to generate higher dimensionality in order to aid in assignment of proteins, measured J-couplings may be used to determine the dihedral angles ϕ and χ^1 using the Karplus equations and aid in stereospecific assignments of nonequivalent H_β methylene resonances.

I.3.4. Dipolar Coupling

Simply stated, dipolar coupling is the interaction of the magnetic moments of two spins through space. It also carries the name through-space correlation. The effect of dipolar coupling is called the nuclear Overhauser enhancement (or NOE) where two spins which are close in space can transfer magnetization to each other by a process called cross-relaxation. In a NOESY spectrum, correlations will be observed if two resonances are less than 5 Å apart since the NOE is proportional to r^{-6} where r is the distance between two nuclei. This experiment is the principle source of information used to solve three-dimensional protein structures by NMR spectroscopy.

An example of a 2D NOESY experiment is shown in Figure I.21A. In this scheme, three rf pulses are applied to proton spins. The scheme is repeated while t_1 is successively incremented. The signals measured in t_2 will be modulated by the frequencies present during t_1 according to the length of the NOE mixing period. This experiment will give rise to two time-modulated frequency axes (t_1 and t_2) and a 2D Fourier transform will give rise to a 2D spectrum with axes f_1 and f_2 (Figure I.21B).

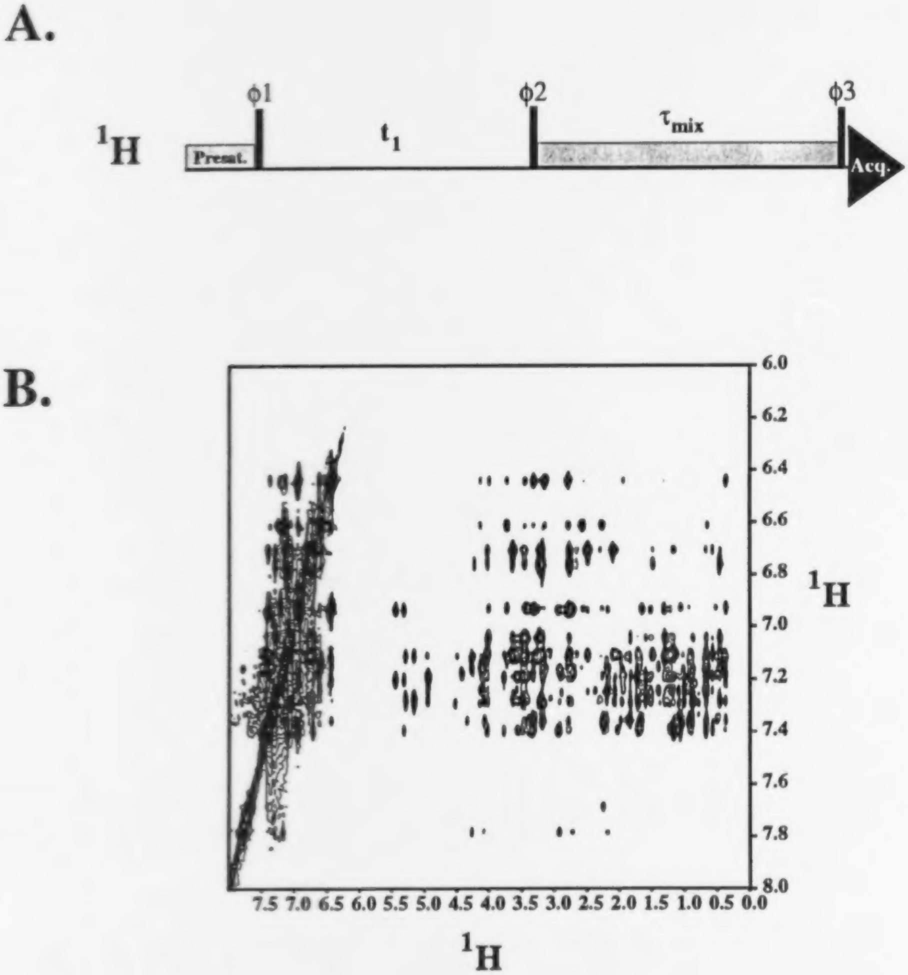

Figure I.21. (A) Pulse scheme of a 2D NOESY experiment. (B) 2D NOESY spectrum of TnC (shown are the aromatic NOE's).

I.3.5. Determination of protein structures.

1.3.5.1 Assignment

The key to solving a protein structure is to assign each resonance and then identify each pairwise through-space NOE interaction. There are two types of experiments that are relied upon to sequentially assign a spectrum: those demonstrating through-bond and those demonstrating through-space correlations.

There are two key through-bond experiments: namely the COSY and the HOHAHA (homonuclear Hartmann-Hahn) or TOCSY experiments. The HOHAHA experiment is versatile as the mixing time may be adjusted to obtain direct, single or multiple relayed connectivities. These experiments generally serve to group together the protons belonging to the same residue.

The first step in assignment, involves identifying a few spin systems based upon COSY or TOCSY data. Using the scalar through-bond 3D triple resonance experiments such as the HNCA (where correlations are made from the ^{1}H and ^{15}N of residue i to the Cα of residue i and i-1), the HN(CO)CA (correlations are made from the ^{1}H and ^{15}N of residue i to the Cα of residue i-1), the HNCO (correlations are made from the ^{1}H and ^{15}N of residue i to the C' of residue i-1), the HCACO (correlations are made from the ^{1}Hα of residue i to its own Cα and C'), and the ^{15}N-edited TOCSY (to obtain the correlation from the ^{1}H and ^{15}N of residue i to its own ^{1}Hα) coupled with a through-space experiment (the ^{15}N-edited NOESY to correlate the ^{1}HN and ^{15}N of residue i with the ^{1}HN of residue i ± 1, as well as the ^{1}Hα of i-1), sequential assignment may be accomplished for a large protein. Side chain assignments could then be assigned using the 3D HCCH-COSY or HCCH-TOCSY experiments. More details of these experiments will be described in chapter II.

If a protein or peptide is not labeled with ^{13}C or ^{15}N, then the combination of 2-dimensional COSY and TOCSY spectra for the identification of spin systems coupled

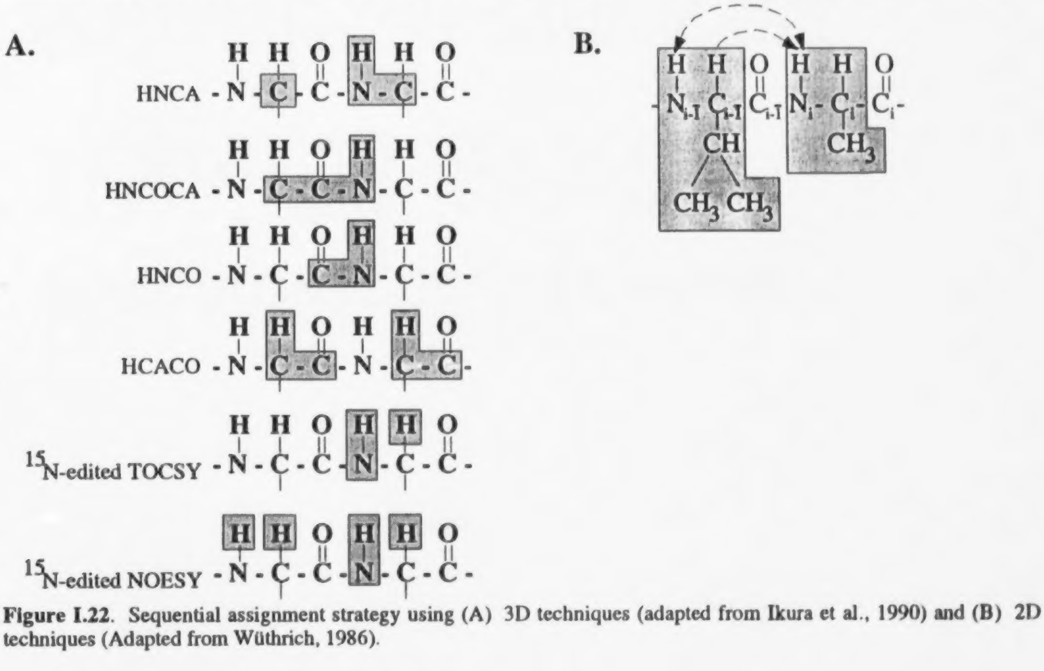

Figure I.22. Sequential assignment strategy using (A) 3D techniques (adapted from Ikura et al., 1990) and (B) 2D techniques (Adapted from Wüthrich, 1986).

with 2D-NOESY d_{NN} and $d_{\alpha N}$ information must be used. An example of the assignment of a peptide using these methods will be given in chapter IV.

Figure I.22 illustrates the sequential assignment strategy for homonuclear and heteronuclear assignment.

I.3.5.2 Secondary structure determination.

Once most of the backbone resonances have been assigned, a detailed study of the backbone NOE's needs to be accomplished. Figure I.23 illustrates the sequential and medium range NOE's from various types of secondary structure. In practice, the information on sequential distances ($d_{\alpha N}$ and d_{NN}) gathered during sequential assignments already provides a strong indication of the sequence locations of helices and β-strands (Wüthrich, 1986). These are confirmed or discarded using the medium range ^1H-^1H distances for helices ($d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ and interstrand long-range distances for β-sheets ($d_{\alpha\alpha}(i,j)$) (Wüthrich, 1986).

^1H, $^{13}C\alpha$, and $^{13}C'$ resonance chemical shifts also contain valuable information. A correlation has been found between the protein backbone angles ϕ and ψ and the $^{13}C\alpha$, $^{13}C_\beta$, $^{13}C'$ and $^1H\alpha$ chemical shifts (Wishart et al., 1991a,b; Wishart and Sykes, 1994; Spera and Bax, 1991). The $^{13}C_\beta$ and $^1H\alpha$ resonances shift downfield from random coil positions when the nuclei are in extended structures, whereas they shift upfield from random coil positions when the nuclei are present in α-helical structures. $^{13}C\alpha$ and $^{13}C'$ follow the opposite trend.

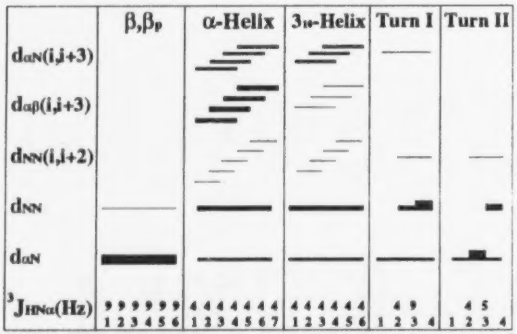

Figure I.23 Survey of the sequential and medium range ^1H-^1H NOE's and coupling constants $^3J_{HN\alpha}$ in various types of secondary structures. Adapted from Wüthrich, 1986.

A new method for characterizing helices is the ratio of the $d_{N\alpha}/d_{\alpha N}$ (Gagné et al., 1994) (where $d_{\alpha N}$ refers to the NOE involving the Hα of residue i-1 to the HN of residue i, and the $d_{N\alpha}$ refers to the NOE between HN of residue i and Hα of residue i). α-helices are characterized by a $d_{N\alpha}/d_{\alpha N}$ ratio greater than one, whereas β-sheets are characterized by a $d_{N\alpha}/d_{\alpha N}$ ratio less than one.

Another way in which to determine the ϕ angle is through the determination of $^3J_{HNH\alpha}$ coupling constants using the Karplus equation. α-helices are characterized by small HN-Hα J couplings due to the smaller angle between the HN and Hα protons whereas β-sheets have large HN-Hα J couplings due to the larger dihedral angle between the HN and Hα protons. A detailed secondary structure analysis of TnC will be shown in chapter VI.

I.3.5.3. Tertiary structure determination.

Once all (or most) NOE's have been identified and torsion angle restraints have been determined (for details see chapter VII), an iterative cycle begins where starting structures are developed and these are used to find more NOE's (Figure I.24). A number of computational strategies may be applied to locate the minimum of a target function which encompasses terms for the experimental restraints, covalent geometry, and nonbonded contacts. Here, the use of the program XPLOR was used to generate 50 structures of TnC. Details of the determination of the structure of TnC will be described in Chapters III and VII.

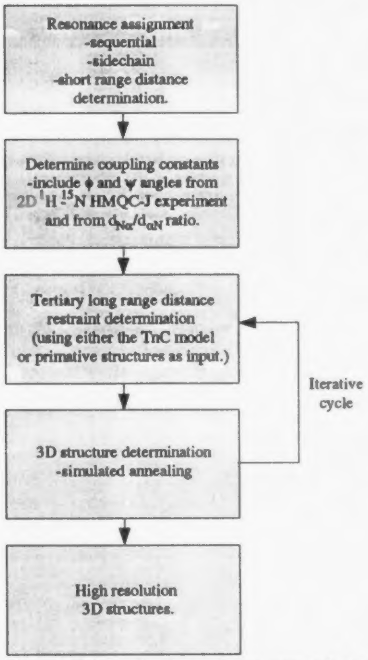
Resonance assignment
-sequential
-sidechain
-short range distance determination.
Determine coupling constants
-include ϕ and ψ angles from 2D ^1H-^{15}N HMQC-J experiment and from $d_{N\alpha}/d_{\alpha N}$ ratio.
Tertiary long range distance restraint determination (using either the TnC model or primative structures as input.)
Iterative cycle
3D structure determination
-simulated annealing
High resolution 3D structures.

Figure I.24 Outline of the general strategy used to determine the 3D structure of TnC.

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Chapter II: *Developments in NMR.*

The determination of protein structures using NMR spectroscopy began in the 1980's. Procedures were developed to analyze spectra (Wüthrich, 1986) using 2D homonuclear NMR. These 2D techniques for solving protein structures were limited to proteins of less than 10 kD mainly due to spectral overlap in 2D space. The most important development in protein NMR was the addition of a third frequency dimension to provide additional resolution. 3D NMR techniques were first introduced eight years ago (Griesinger et al., 1987a,b; Vuister and Boelens, 1987) and have become increasingly popular with new pulse sequences emerging almost weekly.

Three-dimensional spectroscopy is a straight-forward extension of two dimensional spectroscopy where an FID is recorded as a function of the time variable t_3 with two independently incremented time parameters t_1 and t_2 (Griesinger et al., 1987a). The 3D spectrum may be obtained by various combinations of coherence transfer processes. Some of the first 3D experiments were homonuclear (i.e. the 3D-NOESY-NOESY, NOESY-COSY or COSY-NOESY).

With the use of modern molecular biological techniques, isotope labels (^{13}C / ^{15}N) may easily be incorporated into proteins which have been cloned and expressed in bacteria. For large proteins, heteronuclear spectroscopy can be used in combination with homonuclear spectroscopy to achieve a 3D spectrum with even greater resolution than the strictly homonuclear case, since at least one of the coherence transfer steps involves scalar couplings much larger than the ^{1}H linewidths and the number of resonances in a heteronuclear 3D experiment are smaller than for a homonuclear 3D experiment (Fesik and Zuiderweg, 1988). The first heteronuclear 3D experiments were conceived in 1988 by Fesik and Zuiderweg. They described two experiments which were a combination of the 2D heteronuclear multiple-quantum correlation (HMQC) experiment and either the 2D homonuclear NOESY or COSY experiments. These experiments were called the "HMQC-NOESY" and "HMQC-COSY" respectively.

Since these first experiments, many heteronuclear 3D techniques have evolved. Some of these are simply homonuclear NOESY or HOHAHA experiments edited by the attached ^{15}N and/or ^{13}C (Kay et al., 1989a; Marion et al., 1989a,b; Vuister et al., 1992; Palmer et al., 1992; Kay et al., 1990a) or are more exotic such as the highly sensitive triple resonance ^{15}N and ^{13}C correlated experiments derived from one-bond J couplings (Ikura et al., 1990; Bax and Ikura, 1991; Powers et al., 1991; Seip et al., 1992; Grzesiek and Bax, 1992a; Bax and Pochapsky, 1992; Davies et al., 1992; Weisemann et al., 1992; Muhandiram and Kay, 1994). There has also been a surge of techniques correlating sequential backbone assignments with the sidechain assignments (Grzesiek and Bax, 1992b,c; Logan et al., 1992; Kay, 1993a,b; Grzesiek et al., 1993; Wittekind and Mueller, 1993) as well as techniques mainly for sidechain assignment (Kay et al., 1990b; Bax et al., 1990; Ikura et al., 1991a; Emerson and Montelione, 1992). These new techniques have made possible the assignment and structure elucidation of many proteins such as thioredoxin (Forman-Kay et al., 1991), calmodulin in complex with the myosin light chain kinase peptide (Ikura et al., 1992), FK506 binding protein / ascomycin complex (Meadows et al., 1993), cyclosporin A / cyclophilin complex (Thériault et al., 1993), pleckstrin homology domain (Yoon et al., 1994), the amino-terminal fragment of urokinase-type plasminogen activator (Hansen et al., 1994), and now TnC (chapter VII).

As was shown in chapter I, the crystal structure of half-saturated TnC reveals primarily α-helical structure. This poses problems for solving the structure of calcium-saturated TnC using 2D techniques since the ^{1}H spectral dispersion is small for residues in an α-helical conformation, and thus spectral overlap is severe. With the introduction of modern 3D techniques, however, the problem of the severe spectral overlap inherent with large molecules and α-helical structure is somewhat alleviated.

This chapter describes the three-dimensional and two-dimensional techniques needed for the study of the structure of TnC. Many of these pulse sequences were programmed by myself and another graduate student Stéphane Gagné. Those pulse sequences which I implemented appear in Appendix A.

II.1 2D HSQC

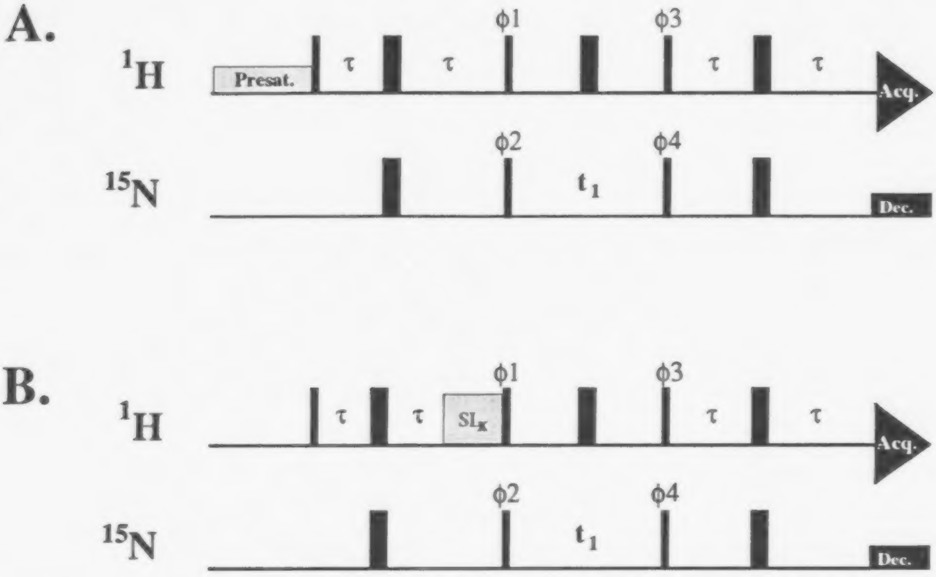

Figure II.1 Pulse sequence of the ^{1}H-^{15}N HSQC experiment using either (A.) presaturation or (B.) spin lock water suppression. The delay times are: $\tau = 1/4J$ (where J is the one-bond coupling constant between the ^{1}H and the attached ^{15}N nucleus (approximately 95 Hz)) and t_1 is the evolution period of the ^{15}N chemical shift. All pulses which are not marked are along x. The phase cycling is as follows: $\phi 1 = 2(y), 2(-y)$, $\phi 2 = x, -x$, $\phi 3 = 4(x), 4(-x)$, $\phi 4 = 8(x), 8(-x)$, Acq. = x, 2(-x), x, 2(-x, 2(x), -x), x, 2(-x), x. Quadrature detection in F_1 is achieved by using the method of States et al. (1982) where the phase of $\phi 2$ is incremented by 90° in alternate scans to generate complex data in t_1. References: Bodenhausen and Ruben, 1980; Messerle et al., 1989.

The 2D ^{1}H-^{15}N HSQC experiment correlates ^{1}H magnetization with its attached ^{15}N nucleus. The experiment was originally proposed by Bodenhausen and Ruben (1980), and uses an INEPT (insensitive nuclei enhanced by polarization transfer) sequence to transfer proton magnetization to its attached ^{15}N nucleus, and back again. The initial INEPT transfer of magnetization results in a gain in sensitivity of the ^{15}N spin of γ_I/γ_S (where I is a proton spin, and S is an ^{15}N spin) due to the initial excitation of the proton (I) spin. For ^{15}N, a factor of 10 is gained in sensitivity, and for ^{13}C, a factor of 4 is gained in sensitivity.

A more rigorous description of this experiment as described in Bodenhausen and Ruben (1980), and Norwood et al. (1990) is as follows. Upon application of the first 90° ^{1}H pulse along x, ^{1}H transverse magnetization is generated along y. In product operator formalism, where proton magnetization is represented by I and ^{15}N magnetization is represented by S, at equilibrium the situation will exist that net magnetization will be along z for both the I and S spins ($I_Z + S_Z$). Upon application of 90°x on the I spins:

$$\gamma_1 I_z + \gamma_S S_z \xrightarrow{90^\circ_x(\mathrm{I})} -\gamma_I I_y + \gamma_S S_z$$

During the evolution period τ (1/4J), the proton magnetization will consist of two vectors precessing with frequencies δH ± (1/2) J_{NH}. The 180° ^{1}H/^{15}N pulse refocuses the chemical shift with no effect on the homo or heteronuclear coupling. At the end of the second τ period, the two vectors refocus along opposite (+x, and -x) axes of the rotating frame (ie. become antiphase). Protons attached to anything other than an ^{15}N nucleus will have their magnetization in-phase along the y-axis. Removing the constant gyromagnetic terms (γ_I and γ_S) to simplify the description, then at the end of the 1/2J period,

$$-I_y \xrightarrow{2\tau} -I_y \cos \pi J_{IS}(2\tau) - 2I_x S_z \sin \pi J_{IS}(2\tau)$$

Only the antiphase term is effective in the polarization transfer, and therefore only this term will be considered in the following discussion. Since ^{1}H magnetization is antiphase along x, the next proton pulse must by shifted 90° relative to the first one. Application of a 90°y ^{1}H proton pulse flips the vectors along z and -z. The two ^{15}N transitions have now become associated with the dramatically enhanced population difference of the protons, and thus application of the 90°x ^{15}N pulse brings the ^{15}N magnetization into the transverse plane along the +y and -y axes.

$$-2I_x S_z \xrightarrow{90^\circ_y(I), 90^\circ_x(S)} 2I_z S_y$$

During the evolution of the ^{15}N chemical shift, a proton pulse is applied which changes the identity of the two ^{15}N vectors to eliminate the coupling between the ^{1}H and ^{15}N nuclei. The second 90°x ^{15}N pulse rotates the y-component back into z generating a population difference which is read by the 90°x proton pulse. The magnetization is refocussed after the application of the second τ delay.

$$2I_z S_y \xrightarrow{t_1} -2I_z S_y \cos(\omega_N t_1) + 2I_z S_x \sin(\omega_N t_1)$$

$$2I_z S_x \sin(\omega_N t_1) \xrightarrow{90^\circ_x(I,S)} 2I_x S_z \sin(\omega_N t_1) \xrightarrow{2\tau} -I_y \sin(\omega_N t_1)$$

$$-2I_z S_y \cos(\omega_N t_1) \xrightarrow{90^\circ_x(I,S)} 2I_x S_z \cos(\omega_N t_1) \xrightarrow{2\tau} -I_y \cos(\omega_N t_1)$$

At this time, a continuous ^{15}N decoupling pulse may be applied during the acquisition period to remove all S-spin coupling.

Figure II.1 illustrates two HSQC pulse sequences using two different types of water suppression, namely presaturation (A), and spin lock (B). Presaturation of water (application of a low power frequency-specific pulse) is undesirable because it obliterates the resonances of rapidly exchanging protons, and via spin diffusion or direct NOE with water, attenuates the entire ^{1}H spectrum. These problems with presaturation may be overcome with the application of the spin lock purge pulses. Basically, the spin lock pulses work because they are applied at the end of the 1/2J period when protons coupled to their directly attached ^{15}N nuclei have $-2I_xS_z$ magnetization (antiphase along x). At this time, all magnetization not bound to ^{15}N nuclei will be in phase along y (I_y). Application of a pulse along x will thus not affect the protons coupled to ^{15}N, but will dephase magnetization present along y and hence water.

Spin-lock water suppression incorporated into the HSQC pulse sequence was able to recover some magnetization (Figure II.2), and was therefore incorporated into several of the 3D experiments. Unfortunately, this type of water suppression is not perfect as the water remains in a semi-saturated state after the end of the pulse sequence since the delay time between scans is typically much shorter than the H_2O T_1 (the T_1 of the H_2O protons is 4 to 5 sec whereas the protein protons have a T_1 around 1 sec). Recently, the lab has acquired a pulse field gradient probe which will allow the water to be unperturbed in a pulse sequence thereby avoiding H_2O saturation. As I have not been involved with developing these pulse sequences, and have not used this probe to acquire any spectra, I will not discuss these novel techniques. They are mentioned here so that the reader is aware that better techniques for water suppression exist. The pulse sequence for the ^{15}N-^{1}H HSQC with spin lock water suppression may be found in Appendix A. All further 3D experiments, with which spin lock was used as the form of water suppression, use essentially the same spin lock pulse as shown for this 2D HSQC case (at the end of the second 1/4J period when proton magnetization coupled to ^{15}N nuclei are antiphase along x ($2I_xS_z$), a spin lock pulse applied along x dephases all magnetization not coupled to ^{15}N which will be present along y).

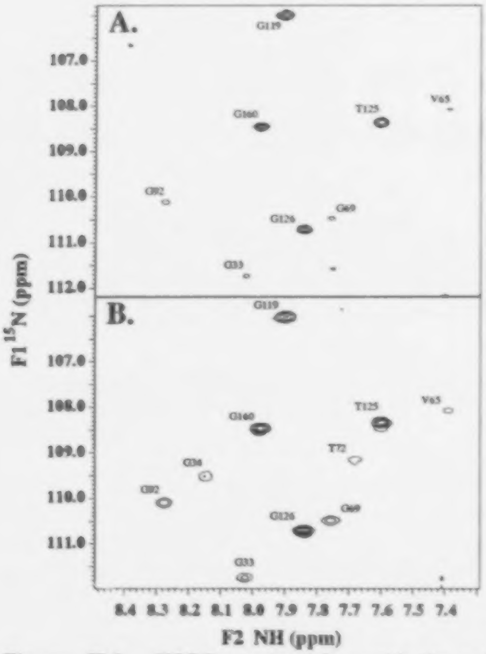

Figure II.2. HSQC spectra taken with the pulse sequences shown in Figure II.1. (A) HSQC with presaturation, and (B) HSQC with spin lock water suppression schemes.

II.2 2D ^{15}N-T$_2$ relaxation

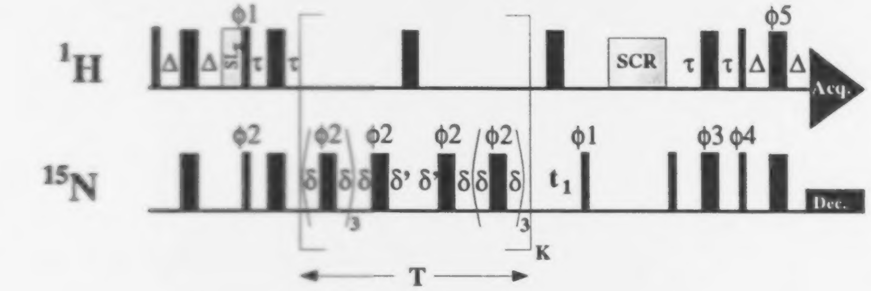

Figure II.3 Pulse sequence used for the measurement of ^{15}N T_2 relaxation times. The delay times are: $\Delta = 2.25$ ms (slightly less than 1/4J to minimize relaxation losses), and $\tau = 2.75$ ms (1/4J), $\delta = 0.45$ ms ($\ll 1/2J$), $\delta' = \delta - pw_{90}$ (^{1}H), and t_1 is the evolution period of the ^{15}N chemical shift. The loop counter K was incremented to lengthen the relaxation delay T. All pulses which are not marked are along x. The phase cycling is as follows: $\phi 1 = y, -y$, $\phi 2 = 2(x), 2(-x)$, $\phi 3 = 16(x), 16(-x)$, $\phi 4 = 8(y), 8(-y)$, $\phi 5 = 4(x), 4(y)$, Acq. = 2(x), 4(-x), 2(x), 2(-x), 4(x), 2(-x). Quadrature detection in F_1 was achieved by using the method of States et al. (1982) where the phase of $\phi 2$ was decremented by 90° in alternate scans to generate complex data in t_1. References: Barbato et al., 1992.

Figure II.3 illustrates the pulse sequence for the measurement of the T_2 spin-spin relaxation rate of ^{15}N nuclei. In essence, the experiment consists of two INEPT type transfers. The first INEPT sequence transfers magnetization from the amide protons to the attached ^{15}N nuclei. This is followed by a CPMG (Carr-Purcell-Meiboom-Gill) sequence for T_2 relaxation, then an evolution period for the ^{15}N chemical shift. A reverse INEPT sequence finally transfers magnetization back to the protons for subsequent acquisition. The CPMG sequence is simply a delay followed by a 180° pulse followed by a delay to refocus the magnetization. The sequence is repeated many times for each experiment allowing magnetization to remain in the transverse plane. The loss of intensity is followed as a function of the time (the length of the CPMG sequence). At the beginning of the ^{15}N T_2 experiment there is an extra 1/2J delay so that the ^{15}N magnetization is in phase at the start of the CPMG portion of the sequence. The CPMG portion of the experiment itself is coded such that cross-correlation between dipolar and CSA relaxation mechanisms may be effectively suppressed as well as helping to stop H_2O recovery during T (this is done by the application of the 180° ^{1}H pulse in the middle of the CPMG sequence which interchanges multiplet components). For a more complete explanation, see Kay et al., (1992). The two scrambling pulses SL$_x$ and SCR are for suppression of H_2O. These pulses remove the need for presaturation which could affect the measurement of the T_2 relaxation times. The pulse program for this sequence may be found in Appendix A.

Analysis of this data is by measurement of the intensity of the crosspeak versus the relaxation time and fitting the curves to a single exponential.

II.3 2D ^{15}N-T_1 relaxation

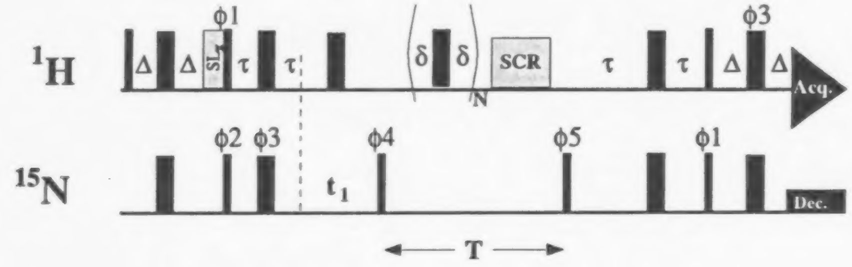

Figure II.4 Pulse sequence used for the measurement of ^{15}N T_1 relaxation times. The delay times are: $\Delta = 2.25$ ms, and $\tau = 2.75$ ms (1/4J), $\delta = 7$ ms, and t_1 is the evolution period of the ^{15}N chemical shift. The loop counter N was incremented to lengthen the relaxation delay T. All pulses which are not marked are along x. The phase cycling is as follows: $\phi 1 = y$, $\phi 2 = 4(x), 4(-x)$, $\phi 3 = 8(x), 8(y), 8(-x), 8(-y)$, $\phi 4 = y, -y$, $\phi 5 = 2(x), 2(-x)$, Acq. = 2(x), 4(-x), 2(x), 2(-x), 4(x), 2(-x). Quadrature detection in F_1 is achieved by using the method of States et al. (1982) where the phase of $\phi 3$ is decremented by 90° in alternate scans to generate complex data in t_1. References: Barbato et al., 1992.

This experiment measures the ^{15}N-T_1 spin-lattice relaxation rate. It is essentially similar to the ^{15}N-T_2 experiment except that the evolution of the ^{15}N chemical shift occurs before the relaxation delay T. After ^{15}N evolution, the in-phase magnetization is pulsed onto the -z axis and magnetization is allowed to relax for time T. Again, the effect of the 180° ^{1}H pulse during the relaxation delay is to suppress the effects of ^{1}H-^{15}N dipolar/CSA cross correlation. The scrambling pulses were applied so that presaturation is not required. The pulse program for this sequence may be found in Appendix A.

Analysis of this data is as for the T_2 relaxation data. References for these two experiments are: Kay et al. (1989b, 1992), and Barbato et al. (1992).

II.4 3D HNCA

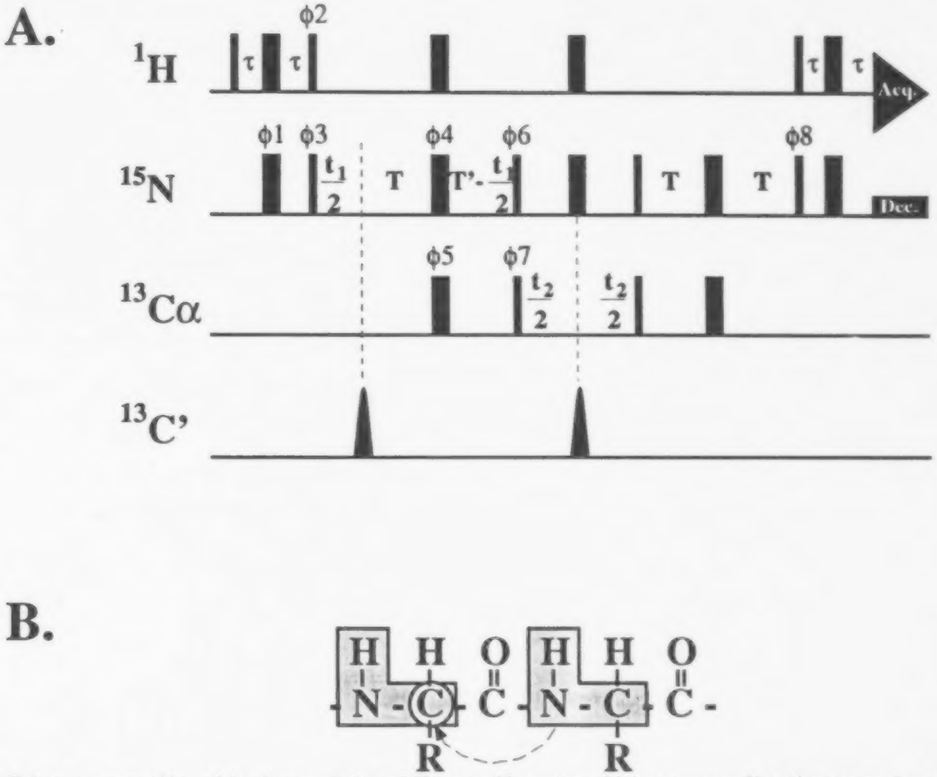

Figure II.5 Pulse sequence (A) used for the correlation of (B) an amide proton with its corresponding nitrogen nucleus to the intraresidue $^{13}C\alpha$ and the interresidue (i-1) $^{13}C\alpha$. The delay times are: $\tau = 1/4J$, t_1 is the evolution period of the ^{15}N chemical shift, and t_2 is the evolution of the $^{13}C\alpha$ chemical shift, T = 13.5 ms, and T' = T + pw180(^{13}C'). All pulses which are not marked are along x. The phase cycling is as follows: $\phi1$ = x, -x, $\phi2$ = y, -y, $\phi3$ = x, $\phi4$ = 4(x), 4(y), 4(-x), 4(-y), $\phi5$ = 16(x), 16(-x), $\phi6$ = 16(y), 16(-y), $\phi7$ = 2(x), 2(-x), $\phi8$ = y, Acq. = 2(x, 2(-x), x, -x, 2(x), -x), 2(-x, 2(x), -x, x, 2(-x), x). Quadrature detection in F_1 and F_2 was achieved by using the method of States et al. (1982) where the phases of $\phi3$ and $\phi7$ were independently incremented by 90° in alternate scans to generate complex data in t_1 and t_2. Relevant coupling constants are: $^1J_{NH} \sim 95$ Hz, $^1J_{NC\alpha} \sim 9\text{-}13$ Hz, and $^2J_{NC\alpha} \sim 5\text{-}10$ Hz. Reference: Grzesiek and Bax, 1992a.

During this experiment, the INEPT sequence transfers longitudinal magnetization of the protons into transverse antiphase magnetization of the ^{15}N nuclei. Evolution due to J coupling and evolution of the ^{15}N chemical shift occurs over the constant time period of total duration 2T (which is an integral multiple of $1/2J_{NH}$). This portion of the experiment causes no decay in the t_1 dimension caused by T_2 relaxation nor any t_1 dependence of dephasing caused by J-couplings (Powers et al., 1991). At the end of this period, the magnetization is still antiphase with respect to its attached proton and is subsequently transferred into antiphase Cα magnetization. The ^{13}Cα chemical shift is then allowed to evolve and the transfer of magnetization back to the amide hydrogens follows a reverse pathway (generation of antiphase ^{15}N magnetization which is transferred back to the ^{1}H by the final reverse-INEPT portion of the sequence).

This pulse sequence was modified from the constant time version of the HN(CO)CA which was modified from the original pulse sequence only by inclusion of the constant time portion. Since this was the final sequence used for gathering data on TnC, it has been included here. The original non-constant time version was programmed by myself and is shown in Appendix A. For this constant time version of the HNCA, I included the option of spin lock purge pulses at the end of the first INEPT period (see the spin lock version of the HSQC experiment) and therefore will also be included in Appendix A.

Another concept which was developed at this time was that of the shifted laminar pulse. As can be seen in Figure II.5, four channels are needed to run the experiment (^{1}H, ^{15}N, ^{13}Cα, and ^{13}C'). The ^{13}Cα atoms resonate at approximately 40 to 70 ppm relative to DSS and 13C' resonate at approximately 170 to 185 ppm relative to DSS. Simply moving the carrier from the center of the ^{13}Cα to the center of the ^{13}C' to pulse the ^{13}C' causes problems with phase coherence. These problems may be allieviated with the use of the shifted laminar pulse first described by Patt (1992). These pulses may be thought of as a series of sequential amplitude and phase modulated rectangular pulses. For example, if we require a pulse to shift its excitation by 18 000 Hz, and the pulse has a duration of 55 μs, then if we apply 275 pulses of 0.2 μs each and successively advance the phase of each of the pulses by 6.48°, the excitation maximum of the pulse is shifted by 6.48°/360° = 18 kHz.

Multiple pulses may also be formed and these are used in the HCCH COSY spectrum where decoupling of ^{13}C' and the ^{13}C of the aromatic rings need occur. These multiple pulses are the vector sum of the frequencies of interest at the times of each element in the waveform. For these multiple pulses, however, the power must be increased or the pulse width must be doubled since dividing the available power into two frequencies reduces the power available at one frequency.

II.5 3D HN(CO)CA

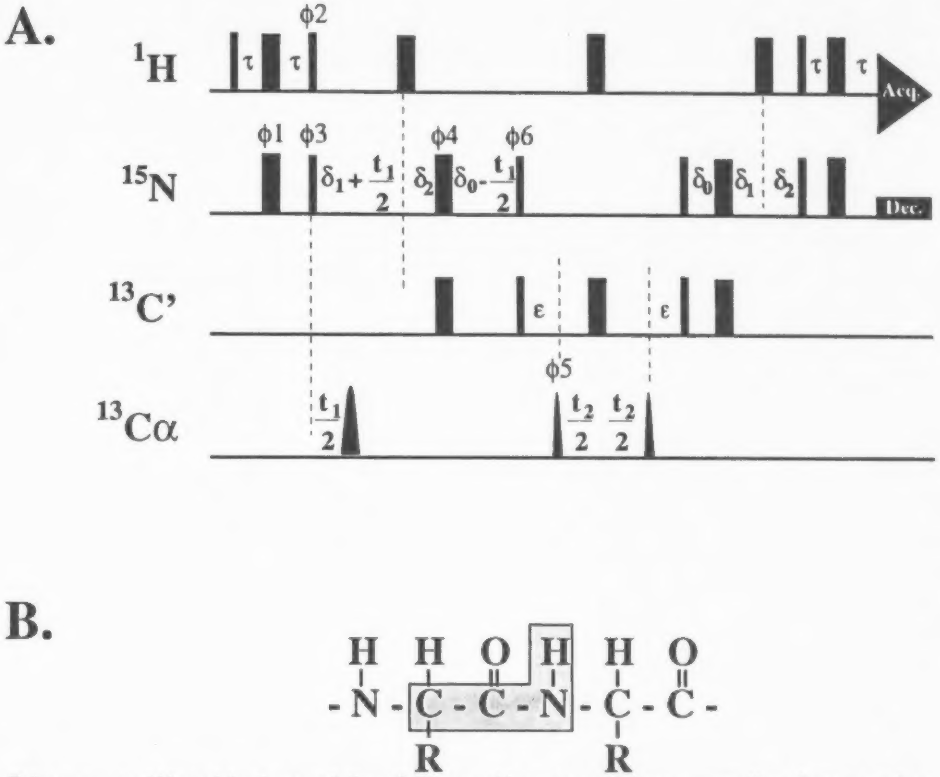

Figure II.6 Pulse sequence (A) used for the correlation of (B) an amide proton with its corresponding nitrogen nucleus to the interresidue (i-1) ^{13}Cα via the intervening ^{13}C' spin. The delay times are: $\tau = 1/4J$, $t_1 = {}^{15}$N evolution, $t_2 = {}^{13}$Cα evolution, $\varepsilon = 5.5$ ms, $\delta_1 = 7.25$ ms, $\delta_2 = 2.75$ ms, $\delta_0 = \delta_1 + \delta_2 + \mathrm{pw}_{C\alpha}180$. All pulses which are not marked are along x. The phase cycling is as follows: $\phi1 = x, -x$, $\phi2 = y, -y$, $\phi3 = x$, $\phi4 = 4(x), 4(y), 4(-x), 4(-y)$, $\phi5 = 2(x), 2(-x)$, $\phi6 = x, -x$, Acq. = 2(x), 4(-x), 2(x). Quadrature detection in F_1 and F_2 was achieved by using the method of States et al. (1982) where the phases of $\phi3$ and $\phi5$ were independently incremented by 90° in alternate scans to generate complex data in t_1 and t_2. Relevant coupling constants are: $^1J_{NH} \sim 95$ Hz, $^1J_{NC'} \sim 15$ Hz, $^1J_{C\alpha C'} \sim 55$ Hz. Reference: Grzesiek and Bax, 1992a.

This pulse sequence (Figure II.6A) correlates a backbone amide hydrogen to its own nitrogen nucleus, and to the interresidue (i-1) ^{13}Cα via the ^{13}C' of the preceding residue (Figure II.6B). The transfer of magnetization in this experiment is similar to what was described for the HNCA experiment. This pulse sequence is important during the assignment of the backbone as it provides sequential information. This sequence using spin lock water suppression may be found in Appendix A, along with the non-constant time version of the HN(CO)CA experiment.

II.6 3D HNCO

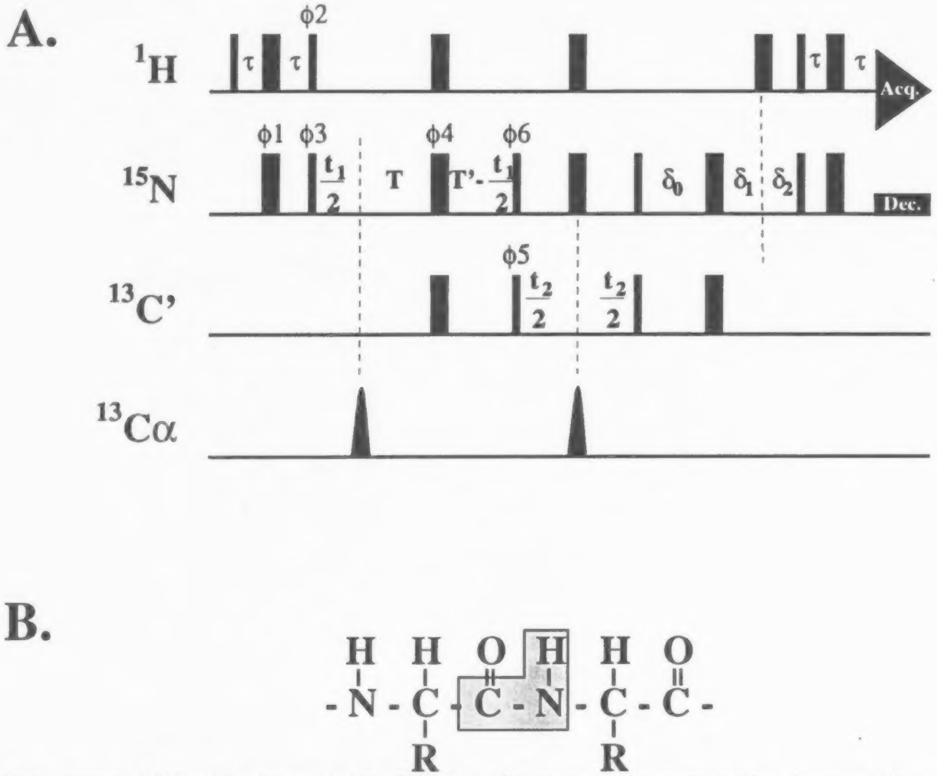

Figure II.7 Pulse sequence (A) used for the correlation of (B) an amide proton with its corresponding nitrogen nucleus to the preceding ^{13}C'. The delay times are: $\tau = 1/4J$, t_1 is the evolution period of the ^{15}N chemical shift, t_2 is the evolution of the C' chemical shifts, T = 13.5 ms, $\delta_1 = 7.25$ ms, $\delta_2 = 2.75$ ms, $\delta_0 = \delta_1 + \delta_2 + pw_C180$. All pulses which are not marked are along x. The phase cycling is as follows: $\phi1$ = x,-x, $\phi2$ = y, -y, $\phi3$ = x, $\phi4$ = 4(x), 4(y), 4(-x), 4(-y), $\phi5$ = 2(x), 2(-x), $\phi6$ = x,-x, Acq. = 2(x), 4(-x) 2(x). Quadrature detection in F_1 and F_2 was achieved by using the method of States et al. (1982) where the phases of $\phi3$ and $\phi5$ were independently incremented by 90° in alternate scans to generate complex data in t_1 and t_2. Relevant coupling constants are: $^1J_{NH} \sim 95$ Hz, $^1J_{NC'} \sim 15$ Hz. Reference: Grzesiek and Bax, 1992a.

Figure II.7 illustrates the pulse sequence of the HNCO experiment. The magnetization transfer for this experiment is similar to that described for the HNCA experiment. The experiment is used for the sequential backbone assignment through the backbone carbonyl carbon (Figure II.7B).

The original non-constant time version of the experiment, as well as the experiment as shown above with the inclusion of the spin lock water suppression scheme are shown in Appendix A.

II.7 3D H(CA)NH

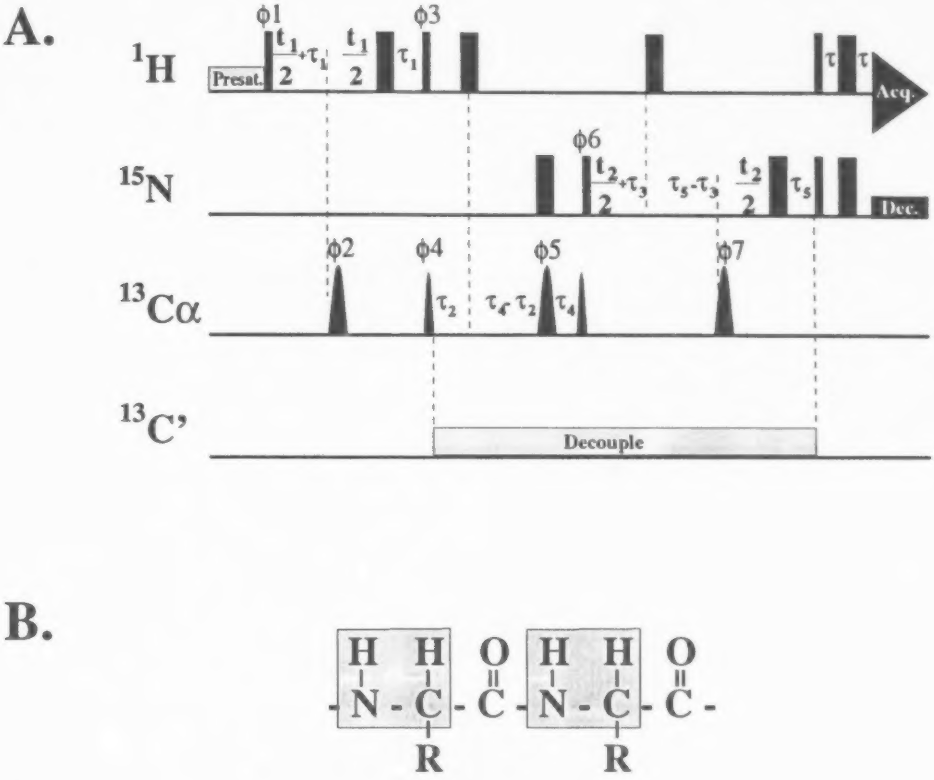

Figure II.8 Pulse sequence (A) used for the correlation of (B) an alpha proton through its attached ^{13}Cα, to an ^{15}N and its attached amide proton. The delays are: t_1 = evolution of ^{1}Hα chemical shift, t_2 = evolution of ^{15}N chemical shift, τ_1 = 1.5 ms, τ_2 = 1.7 ms, τ_3 = 11.5 ms, τ_4 = 12.5 ms, τ = 2.25 ms ($1/4J_{HN}$). The phase cycling is as follows: $\phi 1$ = x, $\phi 2$ = x, -x $\phi 3$ = y, -y, $\phi 4$ = 2(x), 2(-x), $\phi 5$ = 4(x), 4(y), 4(-x), 4(-y), $\phi 6$ = 16(x), 16(-x), $\phi 7$ = 8(x), 8(-x), Acq. = 2(x, 2(-x), x, -x, 2(x), -x), 2(-x, 2(x), -x, x, 2(-x), x). Quadrature detection in F_1 and F_2 was achieved by using the method of States et al. (1982) where the phases of $\phi 1$ and $\phi 6$ were independently incremented by 90° in alternate scans to generate complex data in t_1 and t_2. Relevant coupling constants are: $^1J_{CH}$ ~ 140 Hz, $^1J_{CN}$ ~ 15 Hz, $^1J_{HN}$ ~ 95 Hz. Reference: Kay et al., 1991

This experiment was developed to aid in making the assignment from the ^{1}Hα to the ^{1}HN and ^{15}N nuclei. It was developed before the use of TFE, when an ^{15}N-edited TOCSY experiment revealed very few HN to ^{1}Hα correlations due in part to the secondary structure of the protein and the fact that the linewidths were large because of dimerization (see Chapter V). After the introduction of TFE, however, many of the TOCSY peaks reappeared, and this experiment was no longer needed.

The experiment in Figure II.8A may be easily followed. Hα polarization is allowed to evolve during t_1 and is subsequently transferred via the INEPT sequence to the directly coupled Cα spin. The antiphase Cα magnetization is then transferred to the ^{15}N magnetization via another INEPT type transfer, and evolution of the ^{15}N chemical shift proceeds during t_2. The 180° pulses applied during the evolution of the ^{15}N chemical shift serve to remove the effects of the ^{1}H-^{15}N and ^{13}Cα-^{15}N J coupling. Subsequently, ^{15}N magnetization is refocussed with respect to the ^{13}Cα spin, and transferred to the HN protons via a reverse-INEPT transfer. The original manuscript proposed placing spin lock pulses at the end of the INEPT transfer schemes to remove residual H_2O. In my case, it was found that presaturation worked just as well as spin lock water suppression, and consequently is shown here.

This pulse sequence was not used in the final stages of the assignment of TnC since suitable spectra could be obtained (in the presence of 15% TFE) with the use of the ^{15}N-edited TOCSY. Further, this pulse sequence was not robust. The signal to noise ratio was low, due mainly to starting the pulse sequence on the ^{1}Hα (since the gain in sensitivity for the INEPT transfer is 2.5 times less when the transfer is from ^{1}H to carbon versus ^{1}H to nitrogen), and the fact that at the time of running the experiment the molecular weight of the dimer would have been 36 000 which resulted in very broad lines. Since this experiment was developed and written, it is included here. The pulse program may be found in Appendix A.

II.8 3D HCCH-COSY

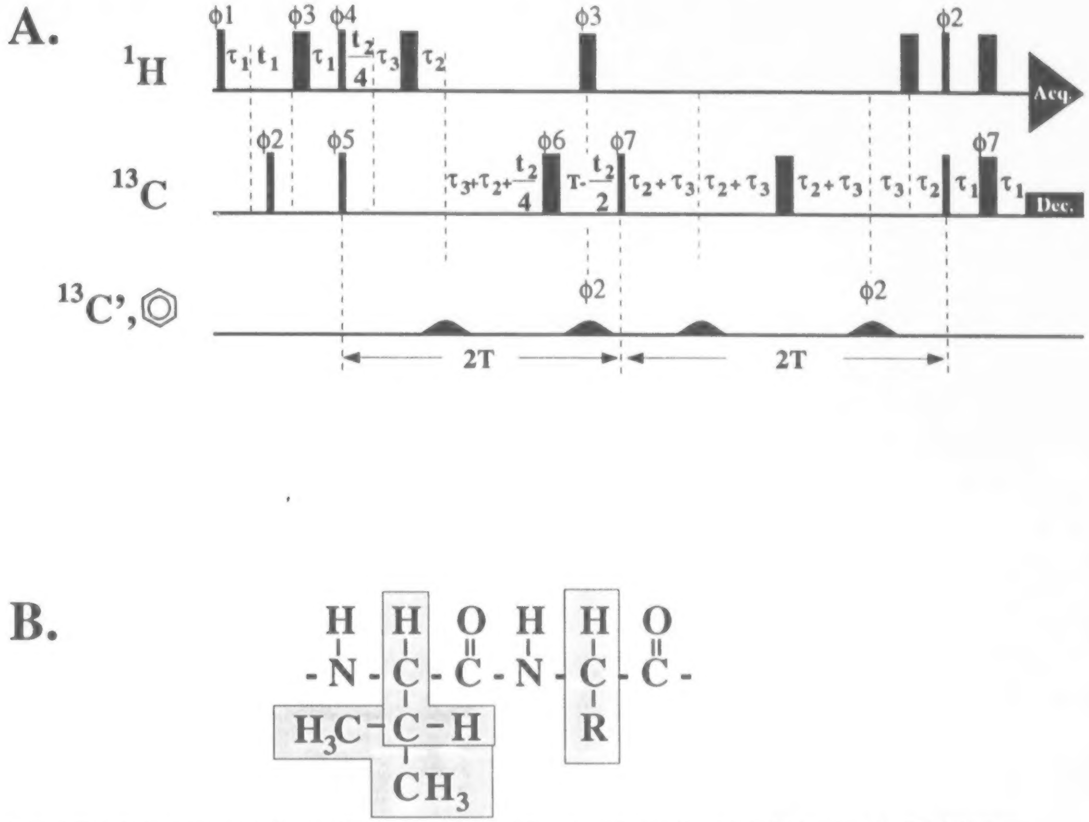

Figure II.9 Pulse sequence (A) used for the correlation of (B) sequential carbon and hydrogen nuclei. The delays are: $\tau_1 = 1.6$ ms, $\tau_2 = 1.1$ ms, $\tau_3 = 0.85$ ms. The duration of the constant time period is 3.9 ms Unless otherwise specified, all pulses are along x. The phase cycling is as follows: $\phi 1 = x$, $\phi 2 = 8(x), 8(-x)$ $\phi 3 = 4(x), 4(y), 4(-x), 4(-y)$, $\phi 4 = y, -y$, $\phi 5 = x$, $\phi 6 = 2(x), 2(y), 2(-x), 2(-y)$, $\phi 7 = 4(x), 4(-x)$, Acq. = x, 2(-x), x, -x, 2(x), 2(-x, 2(x), -x, x, 2(-x), x). Quadrature detection in F_1 and F_2 was achieved by using the method of States et al. (1982) where the phases of $\phi 1$ and $\phi 5$ were independently incremented by 90° in alternate scans to generate complex data in t_1 and t_2 respectively. The shaped 180° pulses were applied simultaneously to the carbonyl carbon and aromatic carbon region of the spectrum. Reference: Ikura et al., (1991b). Relevant coupling constants are $^1J_{HC} \sim 140$ Hz, $^1J_{CC} \sim 55$ Hz.

The sequence presented in Figure II.9 is the sequence used for the assignment of the sidechain carbon and hydrogen resonances. The magnetization transfer is essentially as follows. Magnetization initially on a proton resonance is transferred via an INEPT sequence to the attached carbon while evolving the chemical shift of the hydrogen. The carbon chemical shift is then allowed to evolve while the couplings to backbone or sidechain carbonyls or couplings to aromatic carbons are removed. The time for this (2T) is kept to 7.8 ms. During the next 2T stage, magnetization is transferred to the adjacent carbon nuclei, and finally to their attached protons which are subsequently read during acquisition. The pulse program for the 3D HCCH-COSY is shown in Appendix A.

II.9 4D $^{13}C/^{15}N$-edited NOESY

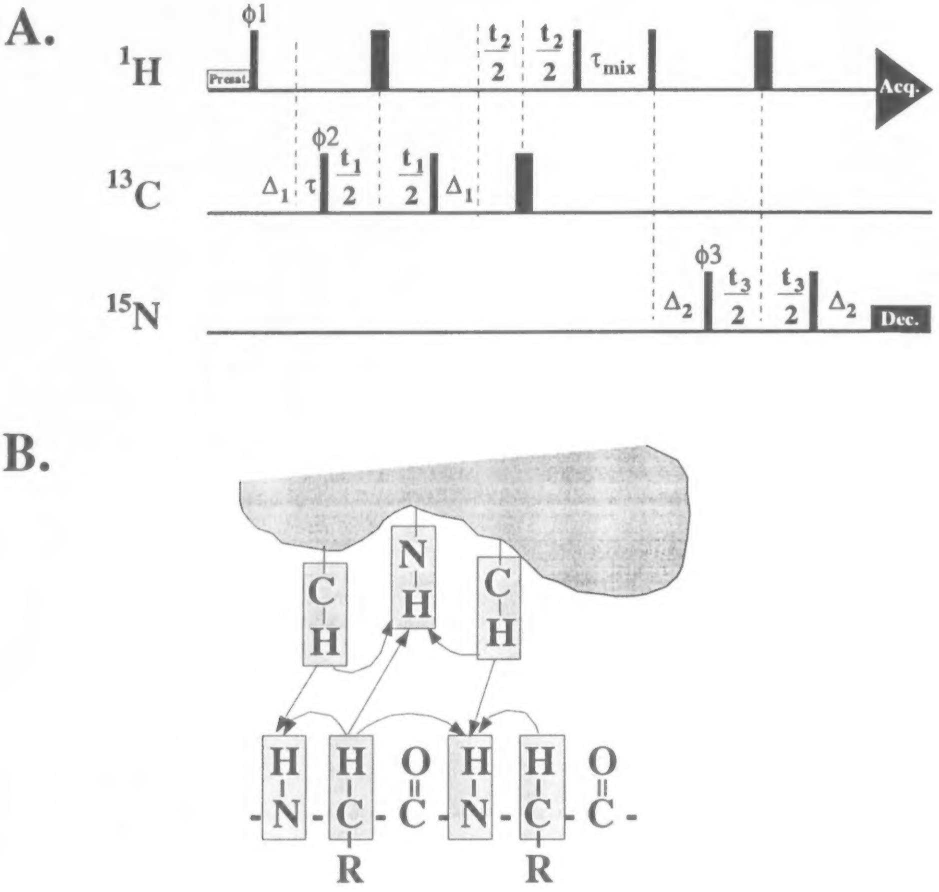
A.
^1H
$\phi1$
Presat.
$\frac{t_2}{2}$
$\frac{t_2}{2}$
τ_{mix}
Acq.
^{13}C
$\phi2$
Δ_1
τ
$\frac{t_1}{2}$
$\frac{t_1}{2}$
Δ_1
^{15}N
$\phi3$
Δ_2
$\frac{t_3}{2}$
$\frac{t_3}{2}$
Δ_2
Dec.
B.
C-H
N-H
C-H
H-N
H-C-R
O=C
H-N
H-C-R
O=C

Figure II.10. Pulse sequence (A) used for the correlation of (B) noes between aliphatic protons and amide protons. This experiment is a 4-dimensional ^{13}C-^{15}N-edited NOESY experiment. The NOE's between amide hydrogens and aliphatic hydrogens are spread out in a third dimension by the ^{15}N chemical shift of the amide ^{15}N atom, and in the fourth dimenion by the ^{13}C chemical shift of the carbon atom. The delays are: $\Delta_1 = 3.0$ ms, $\Delta_2 = 4.5$ ms (slightly less than $1/2J_{HC}$ and $1/2J_{HN}$ respectively), $\tau_{mix} = 75$ ms, t_1 = evolution of ^1H spins attached to carbon, t_2 = evolution of carbon chemical shift, t_3 = evolution of ^{15}N chemical shift. Unless otherwise marked, all pulses are along x. The phase cycling is as follows: $\phi1 = 4(x)$, $\phi2 = 2(x, -x)$, $\phi3 = 2(x), 2(-x)$, and Acq. = x, 2(-x), x. Quadrature detection was achieved by changing the phases of $\phi1$, $\phi2$ and $\phi3$ by 90° in an independent manner in a States-TPPI method. Reference: Kay et al., 1990a. Relevant coupling constants are: $^1J_{HN} \sim 95$ Hz, $^1J_{HC} \sim 140$ Hz.

This experiment (Figure II.10) correlates proton magnetization, with its attached carbon nucleus, with an amide proton and its attached nitrogen nucleus. This experiment is similar to the 3D-^{15}N-edited NOESY experiment, except this 3D experiment is edited in a fourth dimension by the carbon attached to the aliphatic hydrogen. The experiment is used to aid in the assignment of NOE's used as distance restraints. This pulse program may be found in Appendix A.

II.10. 3D HCACO

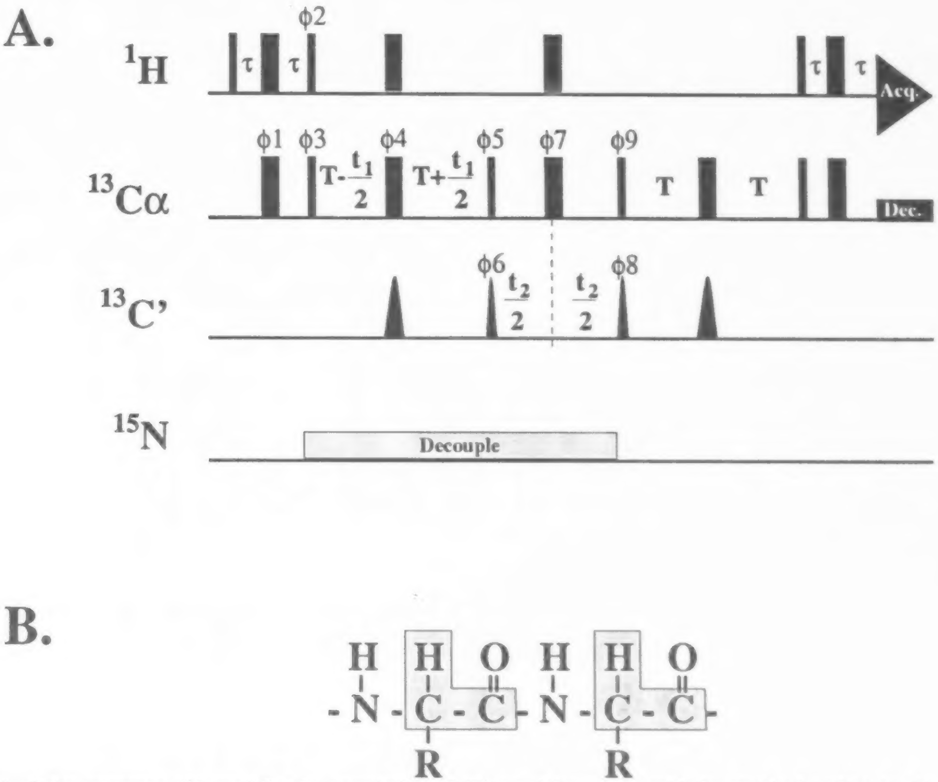

Figure II.11 Pulse sequence (A) used for the correlation of (B) an alpha proton with its attached carbon to the intraresidue carbonyl resonance. The delays are: $\tau = 1/4J_{HC}$, $T = 3.5$ ms, t_1 = evolution of the $^{13}C\alpha$ chemical shift, and t_2 = evolution of the $^{13}C'$ chemical shift. The phase cycling is as follows: $\phi1 = 4(x), 4(-x)$, $\phi2 = y$, $\phi3 = x$, $\phi4 = 8(x), 8(-x)$, $\phi5 = 4(y), 4(-y)$, $\phi6 = x, -x$, $\phi7 = 8(x), 8(-x)$, $\phi8 = 2(x), 2(-x)$, $\phi9 = y$; Acq. = x, 2(-x), x, -x, 2(x), -x. Quadrature detection in F_1 and F_2 was achieved by using the method of States et al. (1982) where the phases of $\phi3$ and $\phi6$ are independently incremented by 90° in alternate scans to generate complex data in t_1 and t_2. Relevant coupling constants are: $^1J_{CH} \sim 140$ Hz, $^1J_{C\alpha C} \sim 55$ Hz. Reference: Powers et al., 1991.

The experiment illustrated in Figure II.11 was used for the sequential backbone assignment to obtain the intraresidue backbone proton - carbon correlations. This experiment is best used in combination with the HNCO and HNCA experiments to obtain the $^1H\alpha$ intraresidue correlation with the $^{13}C\alpha$, and $^{13}C'$.

II.11 3D ^{15}N-edited NOESY

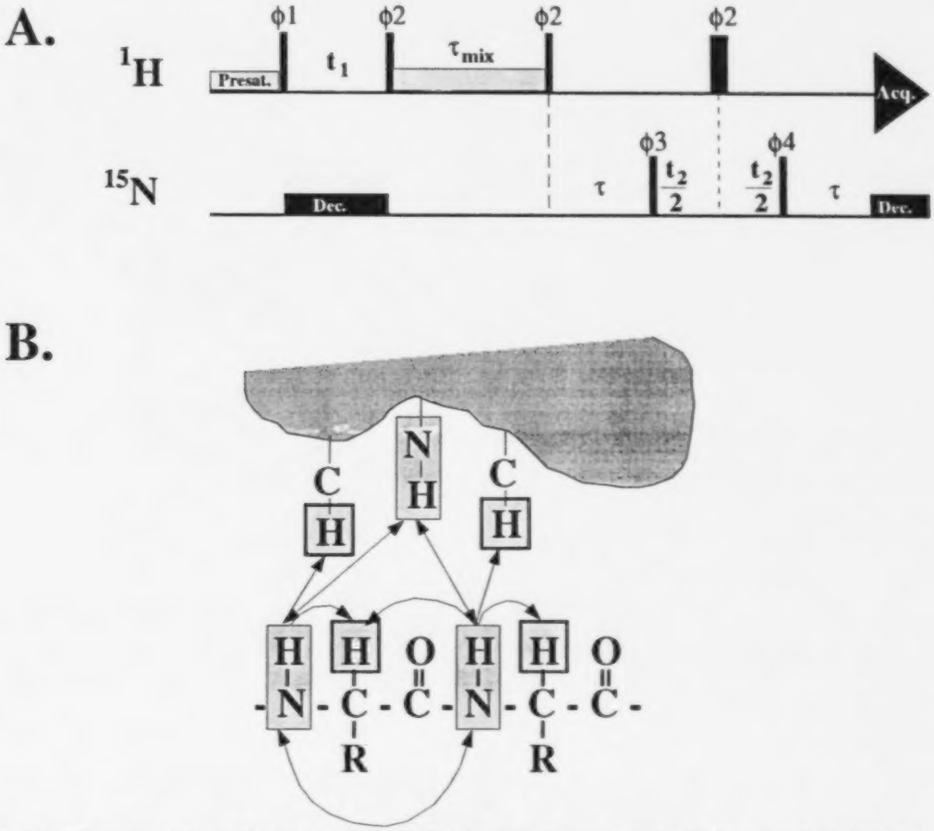

Figure II.12. Pulse sequence (A) used for the correlation of (B) the noe between an amide hydrogen and any proton in the protein. The delay times are: $\tau = 1/2J_{NH}$, $\tau_m = 75$ ms (NOESY mixing time), t_1 = evolution of the proton chemical shift, t_2 = evolution of ^{15}N chemical shift. The phase cycling is as follows: $\phi1 = 2(x, -x), 2(y, -y)$, $\phi2 = 4(x), 4(y)$, $\phi3 = 2(x), 2(-x), 2(y), 2(-y)$, Acq. = x, 2(-x), x, y, 2(-y), y. Quadrature detection in F1 and F2 is achieved by independently altering the phases of φ1 and φ3 by 90° in alternate scans. $^1J_{NH} \sim 95$ Hz. Reference: Kay et al., 1989a.

The pulse sequence shown in Figure II.12 is useful for the sequential backbone assignment (d_{NN}), for secondary structure determination ($d_{N\alpha}$, $d_{\alpha N}$, $d_{\alpha N(i,i+3)}$), and for tertiary structure determination (sequential, medium, and long range NOE's calibrated to generate distance restraints). This experiment was also run as a ^{13}C-edited NOESY experiment to aid in sidechain assignment, and for tertiary structure determination in a similar manner that the ^{15}N-edited NOESY was used.

II.12 3D ^{15}N-edited TOCSY

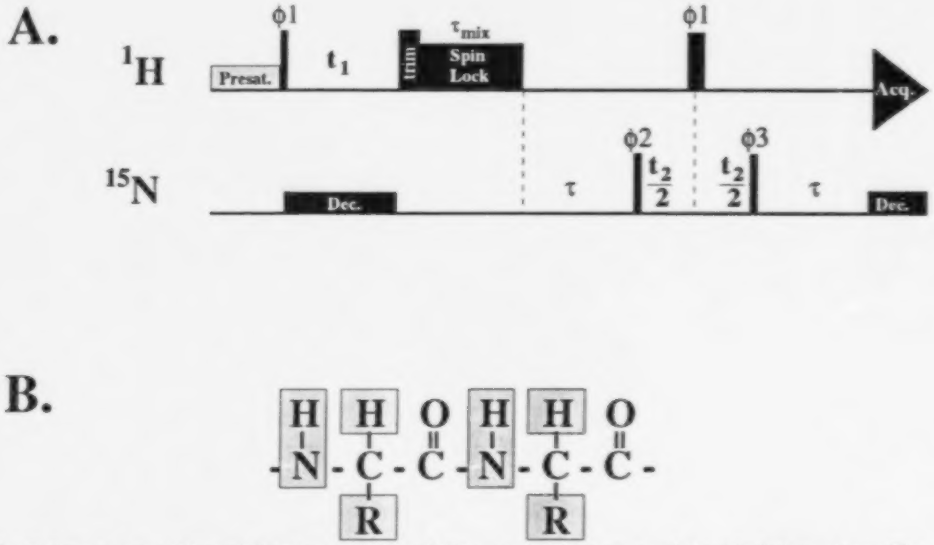

Figure II.13. Pulse sequence (A) used for the correlation of (B) an amide hydrogen and its sidechain protons. The delay times are: $\tau = 1/2J_{NH}$, $\tau_m = 70$ ms (TOCSY mixing time), t_1 = evolution of the proton chemical shift, t_2 = evolution of ^{15}N chemical shift. The phase cycling is as follows: $\phi1 = 2(x), 2(y)$, $\phi2 = x, -x, y, -y$, $\phi3 = 2(x), 2(y)$, $\phi4 = x, -x, y, -y$, Acq. = 2(x), 2(y), 2(-x), 2(-y). Quadrature detection in F1 and F2 is achieved by independently altering the phases of $\phi1$ and $\phi2$ by 90° in alternate scans. $^1J_{NH} \sim 95$ Hz. Reference: Marion et al., 1989b.

The purpose of the experiment in Figure II.13 is for assignment of the backbone amide hydrogen to its Hα, and sidechain residues. The experiment produces a three-dimensional TOCSY spectrum where a 2D ^{1}H-^{1}H TOCSY is edited by an attached ^{15}N chemical shift. Due to the high helical content of TnC, this experiment did not always yield assignment of the Hα resonance.

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Chapter III.

Experimental Methods.

This chapter is a detailed description of the experimental methods used in the remaining chapters. The first two sections will describe how TnC was prepared from muscle tissue, and from an over-expressing plasmid in bacteria. The following section will deal with the synthesis of a 34-residue peptide representing site III of TnC. Next, alkylation of these proteins will be described using two types of reagents followed by the experimental protocols used for the ultracentrifugation and circular dichroism studies of chapter V, as well as a detailed account of the calculation of the interaction affinity between TnC and TnIp which is discussed in chapter VIII. Finally, this chapter will conclude with a description of how the samples were prepared for NMR studies, data acquisition parameters, and details on the methodology used for calculating the structure of TnC.

III.1 Preparation of TnC from muscle tissue.

TnC, isolated from turkey breast tissue, was used in the experiments performed in chapter VIII and was prepared following the procedures of Byers and Kay, 1982; McCubbin et al., 1982; and Eisenberg and Kielley, 1974.

To extract TnC from muscle tissue, troponin was initially isolated from an acetone powder. This procedure involved grinding turkey breast muscle, and extracting for 20 minutes using five volumes of Gube-Straub solution (0.3 M KCl, 0.15 M K_3PO_4, and 0.5 mM ATP, pH 6.5). After centrifugation for 15 minutes at 3000 rpm, the residue was then washed with 10 volumes of H_2O for 20 minutes. Upon centrifugation again (15 minutes at 3000 rpm), the residue was washed with one volume of 95% ethanol at -20° C and the resulting material was squeezed through two layers of cheesecloth. This procedure was repeated three times. The residue was then washed with one volume of acetone at -20°C, and squeezed dry through cheesecloth. This procedure was also repeated three times. The resulting material was then allowed to dry overnight.

Crude troponin was extracted from the acetone powder in the following manner. Using 20 volumes of a buffer consisting of 0.6 M LiCl, 50 mM Tris-HCl, 1 mM DTT, and 0.01 mM $CaCl_2$ at pH 8.0, the acetone powder was suspended and stirred overnight at 4°C. The resulting mixture was then centrifuged at 4100 rpm for 10 minutes, and the pellet was again suspended in 10 volumes of the above buffer and stirred for 2 hours at 4°C. Upon centrifugation again, the supernatants were combined and the pellet was discarded. The pH of the solution was lowered to 4.6 with the addition of 2 M sodium acetate at pH 4.6. The resulting mixture was then centrifuged for 15 minutes at 8000 rpm. The pellet was subsequently dissolved in a saturated Tris solution, and the pH was lowered again by the addition of 2 M sodium acetate to pH 4.6. The resulting mixture was centrifuged at 8000 rpm, and the two supernatants were combined. Following this, 84 mg/L of $NaHCO_3$ and additional Tris were added to the supernatant to keep the solution at pH 7.3. Troponin was subsequently isolated using ammonium sulfate fractionations. The first fractionation (0 - 36%) was done by adding 205.7 g/L of $(NH_4)_2SO_4$ at 4°C. The mixture was centrifuged for 15 minutes at 8000 rpm. The supernatant was then subjected to a 36% to 65% $(NH_4)_2SO_4$ fractionation (400.3 g/L $(NH_4)_2SO_4$ - amount added in first fractionation) at 4°C. The resultant was then centrifuged for 15 minutes at 8000 rpm. Crude troponin, found in the pellet, was then dissolved in 50 mM Tris-HCl, 2 mM DTT, and 0.01 mM $CaCl_2$ pH 7.5 and dialyzed against 20 mM NH_4HCO_3. The resulting dialysate was then lyophilized.

Purification of TnC from the crude troponin followed four columns. Crude troponin (containing tropomyosin), dissolved in column buffer, was loaded onto a hydroxylapatite column (Eisenberg and Kielley, 1974) which had been equilibrated with 1 M NaCl, 1 mM Na_3PO_4, and 2 mM DTT. Pure troponin was then eluted with a linear phosphate gradient from 1 mM to 0.2 M. The troponin fraction was collected and dialyzed versus 20 mM NH_4HCO_3, followed by lyophilization. TnC was purified from troponin by applying troponin dissolved in column buffer to a DEAE sephacel column equilibrated at room temperature with 50 mM Tris HCl, 3 mM EDTA, 1.5 mM DTT and 8 M Urea at pH 7.5. TnC was eluted with a gradient of 0 - 0.55 M NaCl in the above buffer. Crude TnC was then isolated (using UV spectroscopy as a monitor), dialyzed versus NH_4HCO_3 and lyophilized. The final purification step of TnC involved using a DEAE sephacel column as above in the absence of urea. Pure TnC (free from other proteins) was subsequently dialyzed versus NH_4HCO_3 and lyophilized.

To remove calcium and other possible impurities, TnC was dissolved in 0.5 M EDTA at pH 8.0 and purified on a Sephadex G25 size exclusion column equilibrated with 25 mM NH_4HCO_3. The purity of TnC was determined to be > 98% pure using UV spectroscopy, reversed phase HPLC, and SDS-polyacrylamide gel electrophoresis. The overall yield of TnC from approximately 2 kg of turkey breast was 200 mg.

III.2 Preparation of TnC from E. coli.

TnC prepared using a bacterial vector was used for experiments performed in chapters V, VI and VII, and was prepared as follows.

Chicken skeletal muscle TnC was expressed in E. coli using either of two vectors both based on the pET3a system of Studier et al. (1990). The construction of the first of these (pET3a-TnC) has been described by Quaggio et al. (1993). The second (pET3a-TnC*), a smaller version of pET3a-TnC, was constructed from pLcII-Fx-TnC (Reinach and Karlsson, 1988), as a template using the polymerase chain reaction and two oligonucleotides. One of these was designed to include codons for amino acid residues 1-6 (underlined) of the sequence of TnC flanked on the 5' side by nucleotides of the pET3a vector including the NdeI restriction enzyme site (GAG ATA TAC ATA TGG CGT CAA TGA CGG ACC). The second corresponded to codons for amino acid residues 159 - 162 of the non-coding strand of TnC (underlined) flanked on the 5' side by a stop codon and the BamH1 restriction enzyme site and nucleotides of the pET3a vector (GGA ATG TCT GGA TCC TTA CTG CAC ACC CTC). Following the polymerase chain reaction, the amplified DNA fragment was digested with the restriction enzymes NdeI and BamH1 into the NdeI-BamH1 sites of expression vector pET3a plasmid DNA (Studier et al., 1990). (The pET3a-TnC vector was a gift from Dr. Fernando Reinach, and the pET3a-TnC* vector was a gift from Dr. Murali Chandra in Dr. Lawrence Smillie's laboratory.) Expression of TnC protein was in E. coli strain BL21(DE3) pLysS.

Unlabeled TnC was prepared by growing this strain of E. coli in LB medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, pH 7.5 containing 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol). For preparation of ^{15}N-labeled or $^{13}C/^{15}N$-labeled protein, 50 mL of ZB medium (1% N-Z-amine A caesin enzymatic hydrolysate + 0.5% NaCl) (Studier et al., 1990) containing 100 μg/mL ampicillin + 25 μg/mL chloramphenicol, was inoculated with a fresh colony and allowed to grow until an OD_{600} of 0.6 to 0.9 was reached. This culture was then used to inoculate 1 L of minimal media which consisted of M9 salts without NH_4Cl (as described by Maniatis et al. (1986)), 5g of D-glucose or 3g of $^{13}C_6$-D-glucose (for ^{13}C-labeled protein), and 1g of $(^{15}NH_4)_2SO_4$ each of which were dissolved in water and filter sterilized for use in the medium. To enhance the expression level, a mixture of vitamins and minerals was added to the minimal medium. 1 mL/L of a filter sterilized mixture of vitamins (at pH 7.5) (0.1g/100 mL of biotin, choline-chloride, folic acid, niacinamide, D-pantothenate, and pyridoxal, 0.5g/100 mL thiamine, and 0.01g/100 mL of riboflavin in H_2O) (Venters et al., 1991; Hoffman and Spicer, 1991); 1mL/L of a filter sterilized mixture of minerals (2M $MgSO_4$ (Hoffman and Spicer, 1991), 1 mM $FeCl_3$, and 25 mM $ZnSO_4$ (Venters et al., 1991)); 1 mL/L of a filter sterilized solution of 100 mM $CaCl_2$ (Hoffman and Spicer, 1991); 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. The cells were then allowed to grow until an OD_{600} between 0.5 and 1.0 was reached and filter sterilized IPTG was added to a final concentration of 1% to induce TnC production. The cells were allowed to grow for a further 3.5 hours at which time they were centrifuged and french pressed. Pure TnC was recovered using either DEAE sephacel containing urea as outlined by Golosinska et al.(1991), and the same column without urea, or a phenylsepharose column (elution of TnC with EDTA). To remove calcium and other possible impurities, TnC was dissolved in 0.5 M EDTA at pH 8.0 and purified on a Sephadex G25 size exclusion column equilibrated with 25 mM NH_4HCO_3. The purity of TnC was determined to be > 98% pure using UV spectroscopy and reversed phase HPLC. The overall yield of TnC was approximately 50 mg per litre of culture.

III.3 Synthesis of the SCIII sTnC (93 - 126) peptide.

SCIII, prepared using solid phase peptide synthesis procedures, was used for experiments in chapter IV and was prepared as follows.

SCIII was synthesized according to the general procedures for stepwise solid-phase synthesis of Hodges et al. (1981, 1988), and has previously been described by Shaw et al. (1991a,b). The amino acids were protected with a Boc group at the α-amino position. The following amino acids were used with the respective protecting groups: cysteine (methylbenzyl), aspartic acid and glutamic acid (benzyl ester), serine and threonine (benzyl), lysine (2-chlorobenzyloxycarbonyl), arginine (p-toluene sulphonyl), and tyrosine (2,6-dichlorobenzyl). The C-terminal residue, glycine 126, was coupled to the BHA resin to obtain a neutral C-terminal amide on each peptide after cleavage of the peptide from the solid support. The next 10 residues were double coupled, except for glutamic acid which was triple coupled, with a Beckman peptide synthesizer (model 990). Each coupling was monitored with ninhydrin to insure that coupling efficiencies were ≥ 99.5%. The first 11 residues coupled to the resin was a kind gift from Dr. Gary Shaw.

Synthesis of SCIII was continued on an Applied Biosystems peptide synthesizer (model 430A) where the remaining residues were double coupled as the preformed symmetric anhydride (except for asparagine and arginine which were coupled as the HOBt active ester, and the glutamic acid residues which were triple coupled). The peptide was acetylated following the coupling of the final residue by using 25% acetic anhydride/dichloromethane. The peptide was then cleaved from the resin by treatment with hydrofluoric acid (20 mL / g resin) containing 10% anisole and 2% dithioethanol (Hodges et al., 1988).

The amino acid sequence for SCIII is:

```
      93   95         100        105        110
Acetyl-K S  E E E L A  N C F R I  F D K N A  D G Y I D
          115       120        125
           I E E L G  E I L R A  T G-amide.
```

The only difference between this peptide and the amino acid sequence of turkey skeletal TnC or recombinant chicken skeletal TnC is that phenylalanine 112 was replaced by tyrosine to aid in the 2D NMR spectral assignment.

50 mg of the crude peptide was purified at one time using reversed-phase high performance liquid chromatography (HPLC) on a Varian 5000 liquid chromatography system utilizing a Waters M-490 variable programmable detector. The peptide was dissolved in 4 mL of approximately 50% TFA and centrifuged to remove particulate matter. The supernatant was then injected onto a semi-preparative SynChropak RP-P reversed-phase C_{18} column (250 mm X 10 mm i.d.) at a flow rate of 2.0 mL/min. The gradient used was 100% A (0.05% aqueous TFA), followed by a linear gradient to 72% A / 28% B (0.05% TFA/acetonitrile) at a rate of 1% B/min for 28 minutes, followed by a linear gradient to 60% A / 40% B at a rate of 0.1% B/min for 120 minutes and ending with a rapid gradient to 100% B (7.5% B/min for 8 minutes). Fractions were taken and analyzed using an automatic Waters WISP 710 autoinjector system. The analytical runs were performed with an Aquapore RP-300 C_8 column at a flow rate of 1 mL/min. A linear AB gradient (where eluent A is 0.05% aqueous TFA and eluent B was 0.05% TFA/CH_3CN) was used to elute the peptide from the column. The retention time of the crude peptide

when this gradient was used at 1%B per minute was approximately 42 minutes. This pure peptide was subsequently analyzed using amino acid analysis to verify the amino acid composition. Figure III.1A is an HPLC trace of the crude SCIII peptide, and Figure III.1B is an HPLC trace of the pure SCIII peptide.

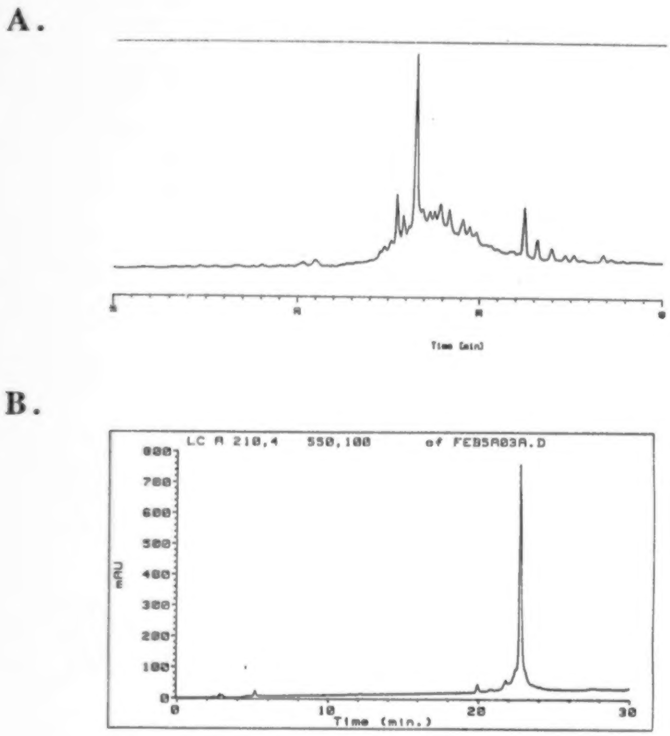

Figure III.1 (A) HPLC run at 2%B/min of crude SCIII. (B) HPLC run at 2%B/min of purified SCIII.

Synthesis of other peptides (used in Chapter VIII): Nα-acetyl-TnI(104-115) amide was synthesized by Paul Semchuk in Dr. Hodges laboratory using the standard procedures for solid-phase peptide synthesis (Erickson and Merrifield, 1976; Talbot and Hodges, 1981; Hodges et al., 1988) on an Applied Biosystems 430A peptide synthesizer (Foster City, California). The synthesis of the site III and site IV peptides, Ac-(A101)(Y112)TnC (93-126) amide (SCIII) and Ac-TnC(129-162) amide (SCIV) respectively of TnC, have been previously described (Shaw et al., 1991a,b).

III.4 Alkylation of TnC and SCIII

For alkylation of SCIII with 3-bromo-1,1,1-trifluoropropanone, the following procedure was used. A buffer consisting of 0.2 M KCl, 0.1 M K_3PO_4 and 21 mM EGTA at pH 7.0 was made and degassed. DTT was added to a final concentration of 20 mM. 10 μL of 3-bromo-1,1,1-trifluoropropanone was added followed (approximately one minute later) by 200 μL of 1 M DTT. 10 μL of concentrated HCl was used to precipitate the peptide. The sample was centrifuged, and the precipitate was brought up into solution by the addition of 400 μL 20 mM NH_4HCO_3 and NaOH (enough of 5 M NaOH to bring pH to approximately 7.0). EDTA was added (100 μL of .05 M) and the sample was desalted on a sephadex G25 size exclusion column equilibrated with 25 mM NH_4HCO_3. Pure TFP-C101-SCIII was pooled and lyophilized in acid-washed containers to prevent calcium contamination. Figure III.2 shows an HPLC spectrum of alkylated SCIII, and a mass spectroscopic analysis of the same.

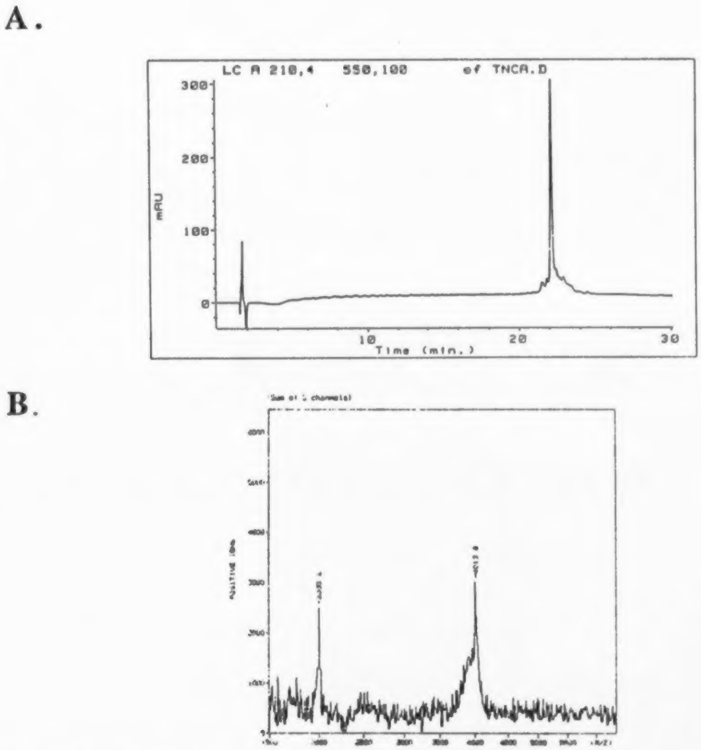

Figure III.2. (A) HPLC run at 2% B/min of TFP-C101-SCIII. (B) Mass spectroscopy analysis of TFP-C101-SCIII.

The general use of 3-bromo-1,1,1-trifluoropropanone (TFP) as an alkylating reagent was discovered to be a problem. If NMR samples were left for any length of time, new peaks would appear in the ^{19}F NMR 1D spectrum. A study of TFP attached to the tripeptide glutathione (γ-Glu-Cys-Gly) revealed that over time, and particularly if oxygen was bubbled through the system, new peaks would appear in a ^{19}F 1D NMR spectrum (Figure III.3A).

TFP-glutathione was prepared by adding a tenfold excess of 3-bromo-1,1,1-trifluoropropanone to glutathione in a buffer consisting of 100 mM KCl, 100 mM Tris-HCl, at pH 7.5 and 25°C. The reaction was allowed to proceed for 15 minutes in the dark and quenched with a 200 fold excess of DTT. Labeled glutathione was then recovered from a sephadex G-10 size exclusion column equilibrated with 25 mM NH_4HCO_3, and lyophilized. A 5 mM sample of S-TFP-Cys-glutathione or unlabeled glutathione was dissolved either in H_2O or D_2O at a pH of 7.0 and 1D ^1H NMR spectra were taken using a Varian VXR-500 NMR spectrometer. A 3.5 mM sample of TFP-glutathione was also prepared in a buffer consisting of 25 mM PIPES, 1 mM TFA in D_2O at a pH of 7.0. This sample was used for the collection of 1D ^{19}F NMR spectra on a Varian Unity 300 NMR spectrometer. Spectra were taken at time 0, 24 hours, 48 hours, 120 hours, 120 hours + 1 hour of air bubbled through the sample, 120 hours + three hours of air bubbled through the sample, and 120 hours + 17 hours of air bubbled through the sample.

The ^1H NMR spectrum of S-TFP-Cys-glutathione revealed that in D_2O, at pH 7, the protons on the methylene carbon of TFP were unobservable (Figure III.3B). It was therefore reasoned that the combined electronegativity of the fluorine group on TFP and the sulfur group on cysteine lowered the pK of the methylene protons of the TFP group to less than 7.0, thereby allowing these protons to exchange with the solvent. The acidity of these protons could cause some sort of undesirable reaction to occur over time thus accounting for the extra peaks in the spectrum.

Due to the instability of the TFP group, the cysteine of TnC was reacted with iodoacetamide to form carboxamidomethylated TnC which remained stable throughout all of the experiments. The method for reacting TnC with iodoacetamide is as follows.

The single cysteine in TnC was reacted with iodoacetamide to prevent oxidation of TnC. The carboxamidomethylation was performed by dissolving TnC in 0.5 M EDTA, and 100 mM DTT at pH 7.5. Iodoacetamide was added to a final concentration of 100 mM (by first dissolving the powder in H_2O and NaOH), and the reaction was allowed to proceed in the dark for 10 minutes. TnC was then recovered on a sephadex G25 size exclusion column equilibrated with 25 mM NH_4HCO_3. The reaction proceeded easily to greater than 95% as determined by DTNB assay, reversed phase HPLC, and mass spectroscopy (MW (determined) = 18 315) (Figure III.4).

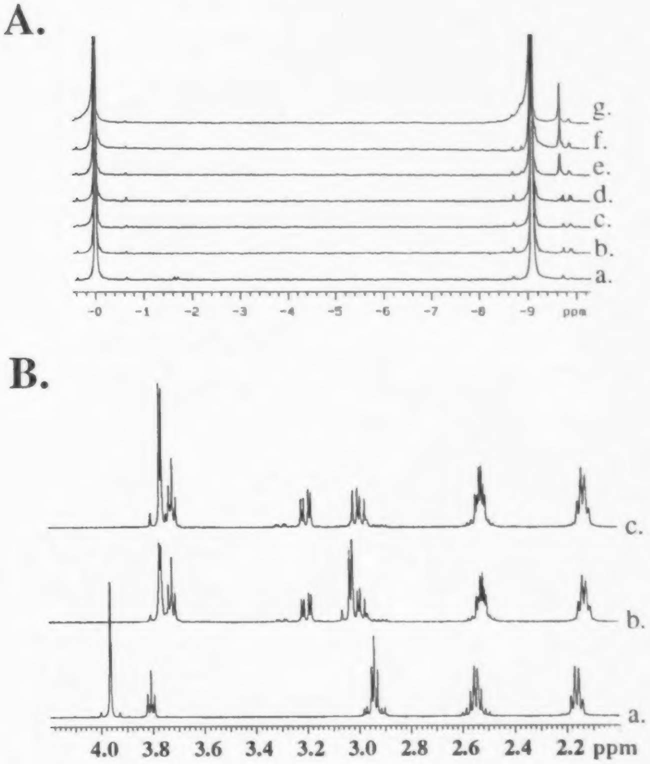

Figure III.3. 5 mM TFP-cys-glutathione. (A) Change in ^{19}F spectra as TFP-glutathione was allowed to oxidize. (a) Spectrum taken immediately upon dissolution of TFP-glutatione, (b) spectrum taken two days later, (c) spectrum taken six days later, (d) same as in 'c' with one hour of oxygen bubbled through, (e) same as in 'c' with 3 hours of oxygen bubbled through, (f) same as in 'c' with 17 hours of oxygen bubbled through. (B) ^{1}H NMR spectra of (a) unlabeled glutathione in D_2O, (b) TFP-glutathione in H_2O, and (c) TFP-glutathione in D_2O.

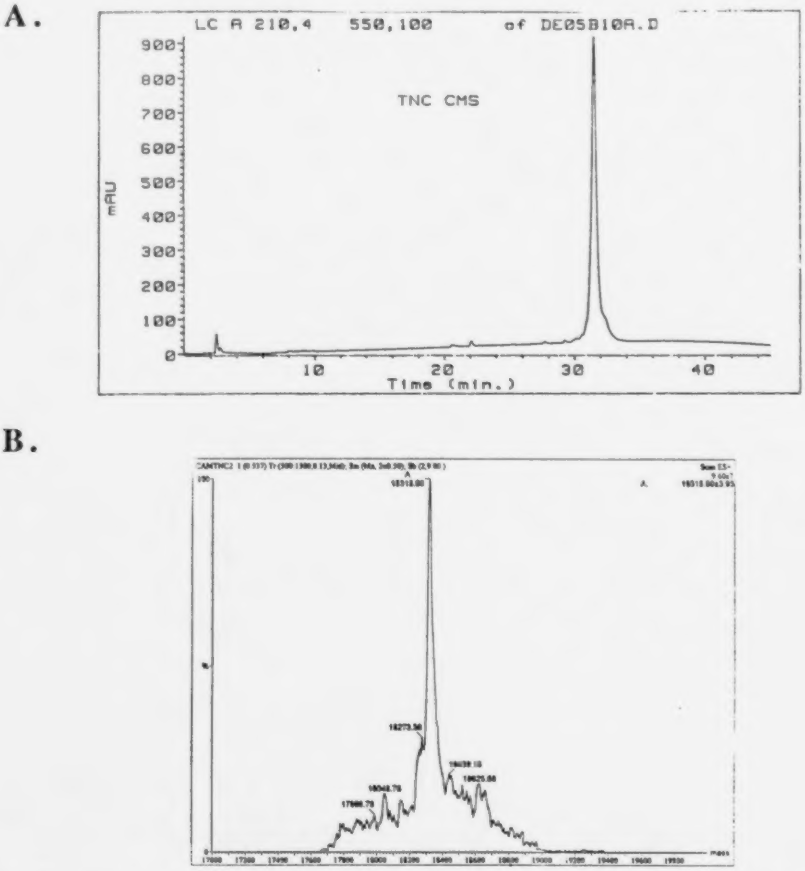

Figure III.4. (A) HPLC analysis of alkylated TnC (run at 2% B per minute) and (B) electrospray mass spectrum of carboxamidomethylated TnC.

III.5 Experimental parameters for other studies.

Ultracentrifugation (Chapter V).

Ultracentrifugation analysis was performed in either 30 mM MOPS, 150 mM KCl, 20 mM $CaCl_2$, and 15% v/v TFE at pH 7.0, or in 30 mM MOPS, 150 mM KCl, and 20 mM $CaCl_2$ at pH 7.0. Both buffers were filtered after preparation. The concentrations of TnC were 0.390 mM in the TFE buffer, and 0.392 mM in the buffer containing no TFE. Both runs were done on a Beckman Model E ultracentrifuge at 16 000 rpm and 20°C employing a photoelectric scanner optical system, where the absorbance was measured at 267 nm and a 1.2 cm path length. All absorbance values were corrected to a 1 cm path length. Molecular weights and dissociation constants were determined by fitting the equation (Burrows et al., 1994):

$$A_{267,r} = c_m \varepsilon_{267} \exp[HM(r^2-r_0^2)] + (c_m c_m/K_d) 2\varepsilon_{267} \exp[2HM(r^2-r_0^2)]$$

The terms of the above equation are: r is the radial distance from the axis of rotation, r_0 is the radial distance at the meniscus, M is the monomer molecular weight, K_d is the dissociation constant for the dimer (K_d = [Monomer]^2/[Dimer]), $A_{267,r}$ is the absorbance at radius r, c_m is the concentration of the monomer at radius r, and ε_{267} is the molar extinction coefficient for TnC at 267 nm which was experimentally determined (after correcting for scatter) to be 0.632 mM^{-1}cm^{-1} for TnC in the absence of TFE and 0.845 mM^{-1}cm^{-1} for TnC in the presence of TFE. The operational constants H and ω are defined by the equations:

$$H = \frac{(1 - \bar{\upsilon}\rho)\omega^2}{2RT} \text{ and } \omega = \frac{2\pi(\text{rpm})}{60}$$

where R is the universal gas constant (8.314 J/mol-K), T is the temperature in K, ω is the angular velocity, ρ is the density of the solution which was 1.0087 g/mL for TnC and 1.0811 for TnC in 15% v/v TFE, and $\bar{\upsilon}$ is the partial specific volume of the solute which is 0.71 mL/g (Byers and Kay, 1982). The partial specific volume may be calculated to give a value of 0.742 mL/g (McCubbin et al., 1985). The value of 0.71 mL/g was used because for highly charged proteins, such as calmodulin, troponin C and S-100, electrostriction of H_2O leads to a lower experimentally determined value for the partial specific volume (Crouch and Klee, 1980; Byers and Kay, 1982; Mani and Kay, 1984). The equation was fit using an iterative non-linear least squares analysis on an in-house written program, where c_m, M, and K_d were allowed to vary to best fit the data points.

CD spectroscopy (Chapter V):

TnC was prepared in 1mL of a buffer containing 50 mM MOPS, 100 mM KCl, and 1mM EGTA with or without 1mM DTT at pH 7.1. To this was added 100 µL of 8M guanidine HCL in 50 mM MOPS buffer, and the resultant TnC solution was dialyzed for 48 hours with two 1L changes of buffer (50 mM MOPS, 100 mM KCl, and 1 mM EGTA). The final concentration of TnC was 0.044 mM. Calcium was then added to a final concentration of 4 mM, to obtain the calcium saturated sample. TFE was added to this sample to obtain the spectrum with 15% v/v TFE. Two runs were averaged to obtain the final far-UV CD spectra, while only one run of the near-UV CD spectrum was taken.

Calculation of Interaction Affinity (Chapter VIII):

Dissociation constants for the reaction P + L ↔ PL were calculated as described previously (Shaw et al., 1991b) using an iterative non-linear least squares analysis. The equation used was

$$K_d = \frac{[P][L]}{[PL]}$$

where [P] is the heterodimer concentration, [L] is the free TnIp concentration and [PL] is the heterodimer - TnIp complex concentration. [PL] was calculated from the total concentrations of P and L according to

$$[PL] = \frac{(P_0 + L_0 + K_d) - \sqrt{(P_0 + L_0 + K_d)^2 - 4P_0L_0}}{2}$$

III.6 Sample preparation.

Protein concentrations were determined from amino acid analysis after hydrolysis in 100-200 µL of 6 N HCl in evacuated sealed tubes for 1 hour at 160 °C. The mean of the molar ratios of all accurately measurable amino acids (alanine and leucine) in the acid hydrolysate were used to calculate the concentration of protein upon comparison to a standard amino acid preparation.

Preparation of samples for use in Chapter IV: For secondary structure determination (2D NOESY, DQF-COSY, and TOCSY experiments), two samples of TFP-C101-SCIII peptide were prepared. The first sample was 1.24 mM TFP-C101-SCIII in a buffer consisting of 50 mM KCl, 1.5 mM $CaCl_2$ at a pH of 7.13 in 80% H_2O, 20% D_2O. The second sample was 1.20 mM TFP-C101-SCIII in a buffer consisting of 50 mM KCl, 1.5 mM $CaCl_2$ at a pD of 7.36 in 100% D_2O (no pH correction for isotope effects). For the 1D ^1H NMR monitored calcium titration, TFP-C101-SCIII was prepared by dissolving lyophilized peptide in one mL of a calcium-free buffer of 50 mM KCl, and 30 mM deuterated imidazole, pH 7.21 to a final concentration of 492 µM. (The buffer was made calcium free by dissolving dithizone to 0.001% in carbon tetrachloride and extracting the buffer until the last two organic extraction phases are green, or by filtering the buffer twice through chelex.) The pH was checked before and after the experiment to ensure it remained at 7.21. Calcium aliquots, which were analyzed by ICP analysis, were added with a carefully calibrated Hamilton syringe. For the 1D ^{19}F NMR monitored calcium titration, TFP-C101-SCIII was prepared by dissolving lyophilized peptide in a buffer consisting of 25 mM PIPES, 150 mM KCl, 0.5 mM TFA in 50% D_2O at pH 6.70. Before the addition of the peptide, the buffer was made calcium-free with the use of chelex and dithizone treatments. The final concentration of TFP-C101-SCIII was 371 μM. As with the ^1H monitored calcium titration, calcium, the concentration of which was analyzed by ICP, was added with a carefully calibrated Hamilton syringe.

Preparation of samples for use in Chapters V to VII: Chicken recombinant TnC was cloned and expressed as described above. Four TnC samples were prepared. The first sample was prepared by dissolving lyophilized ^{15}N,^{13}C-TnC in a buffer consisting of 150 mM KCl, and 15 mM $CaCl_2$ in 99.5% D_2O at pH 7.6. This sample was lyophilized and re-dissolved in 99.5% D_2O three times. After the last lyophilization step, the sample was dissolved in 85% D_2O and 15% TFE and the pH was adjusted with DCl to a pH of 7.0. This sample was used in the 3D HCACO, HCCH-COSY, ^{13}C-edited NOESY and the 2D NH exchange ^1H-^{15}N HSQC experiments. The second sample was prepared by dissolving lyophilized ^{15}N,^{13}C-TnC in a buffer consisting of 150 mM KCl, and 15 mM $CaCl_2$ in 75% H_2O, 10% D_2O, and 15% TFE (for use in the 3D HNCA, HNCO, HN(CO)CA, and 4D ^{13}C-^{15}N-edited NOESY experiments). The third sample was prepared by dissolving lyophilized ^{15}N-TnC in a buffer consisting of 150 mM KCl, and 15 mM $CaCl_2$ in 75% H_2O, 10% D_2O, and 15% TFE (for use in the 3D ^{15}N-edited TOCSY, ^{15}N-edited NOESY, and the 2D HMQC-J, and HSQC experiments). The last sample was prepared by dissolving lyophilized unlabeled TnC in a buffer consisting of 150 mM KCl, and 15 mM $CaCl_2$ in 85% D_2O and 15% TFE (for use in the 2D NOESY experiment of the aromatic region). All samples had a pH of 7.0, a protein concentration of approximately 1.4 mM, and all were carboxamidomethylated at cysteine 101 with iodoacetamide as described above. A small amount of DSS was added to act as an internal standard. The pH of the D_2O samples were not corrected for isotope effects.

Preparation of samples for use in Chapter VIII: The TnC sample for ^1H NMR assignments was 2.0 mM turkey s-TnC, 12 mM $CaCl_2$, and 100 mM KCl dissolved in 650 μl of D_2O at a pD of 6.2. 0.1 mM DSS was added to the sample as a chemical shift standard. 3 mM DTT was also added to the sample to prevent oxidation of the sulfhydryl of s-TnC (C101) and thus prevent formation of intermolecular crosslinks. The SCIII/SCIV sample for the 2D NOESY spectrum was 4 mM SCIII/SCIV, 10 mM $CaCl_2$, 50 mM KCl in D_2O pD 7.2. The TnC sample for the ^1H NMR monitored TnI peptide titration was desalted and made apo as described above. Lyophilized TnC was dissolved in 100 mM KCl, 5 mM $CaCl_2$, 0.1 mM DSS and 3 mM DTT in D_2O pD 6.2 to make a final [TnC] = 0.94 mM. The TnI peptide was dissolved to a final concentration of 30.3 mM in 100 mM KCl, 5 mM $CaCl_2$ in D_2O. Small aliquots of the TnI peptide sample were added to the TnC sample using a carefully calibrated Hamilton syringe. The SCIII/SCIV heterodimer sample for the ^1H NMR TnI peptide titration was composed of 152.2 μM SCIV, 171.4 μM SCIII, 430 μM $CaCl_2$, 50 mM KCl, and 30 mM Imidazole-d_4 pH 7.30. Small aliquots of TnI peptide (11.81 mM in 430 μM $CaCl_2$, 50 mM KCl, and 30 mM Imidazole-d_4 pH 7.30) were added to the 152.2 μM heterodimer sample using a carefully calibrated Hamilton syringe.

III.7 NMR data acquisition.

For spectra collected in chapter IV: The 1D ^1H experiments were carried out on a Varian VXR-500 NMR spectrometer at a temperature of 30°C. The carrier was positioned at the water frequency, and water presaturation was carried out with a 2 second pulse. Typical ^1H NMR acquisition parameters were: spectral width = 6000 Hz, acquisition time = 2 seconds, with 256 transients collected. The spectra were processed using a line broadening value of 0.5 Hz, and the data was zero-filled to 64 K points. The 1D ^{19}F NMR experiments were carried out on a Varian Unity-300 NMR spectrometer operating at 282.2 MHz (^{19}F frequency) at a temperature of 30°C. The carrier was positioned at 0 ppm (on the TFA peak). The spectra were acquired with an acquisition time of 2 seconds, a spectral width of 7000 Hz, and 1024 transients were collected. The spectra were processed using a line broadening value of 2.50 Hz and the data was zero-filled to 64 K points. All 2D experiments were carried out on a Varian VXR-500 NMR spectrometer at a temperature of 15°C. The carrier was positioned at the water frequency, and water was removed from the spectra by the application of a presaturation pulse of either 2.0 or 2.5 seconds duration. All 2D spectra were collected with 256 or 512 X 1024 complex points in F1 and F2 respectively, sweep widths of 6000 Hz in both dimensions and 128 transients per increment. NOESY and TOCSY spectra were processed using cosine-squared weighting functions in both dimensions, and the data in the indirectly detected dimension was zero-filled to 2K data points. DQF-COSY spectra were processed with a sinebell function in F2, and zero-filled to 2K data points.

For spectra collected in chapter V: All 2D ^1H-^{15}N HSQC NMR experiments were carried out on a Varian Unity-600 NMR spectrometer or Varian VXR-500 NMR spectrometer using the pulse scheme of Bodenhausen and Reuben (1980), or using the modified form (water saturation using spin lock) as outlined by Messerle et al., (1989). For these experiments, the ^{1}H dimension was centered at the water frequency. Typical acquisition parameters for these spectra were 128 X 512 complex points in F1 (^{15}N) and F2 (^{1}H) respectively. The proton dimension had a spectral width of 6667 Hz (for experiments on the VXR500 spectrometer) or 8000 Hz (for experiments on the Unity 600 spectrometer) and the ^{15}N dimension had a spectral width of 1500 or 1824 Hz on the VXR500 or Unity 600 NMR spectrometers respectively. The spectra were normally processed using cosine-squared weighting and the ^{15}N dimension was zero-filled to 2K data points. For the TFE titration, the intensities were scaled for dilution effects. For the TFE titration, ^{1}H linewidths were measured from the width of the line at half-height. The digital resolution was 6.5 Hz in the ^{1}H dimension.

2D ^{15}N T_2 relaxation experiments were carried out at 40°C on a Varian Unity spectrometer operating at 600 MHz ^{1}H frequency, using the pulse sequence as described by Barbato et al. (1992) except that quadrature detection in F1 was obtained by the method of States et al. (1982). 128 transients were acquired for each t_1 increment. The ^{1}H carrier was centered at 4.70 ppm on the water frequency, and the ^{15}N carrier was positioned at 119 ppm. The spectral widths used were 8000 Hz in F2 (^{1}H), and 1824 Hz in F1 (^{15}N). 128 X 1024 complex points were acquired for seven different durations of the T_2 relaxation delay, T = 0, 14.14, 28.29, 42.43, 56.58, 84.86, and 141.44 ms. A 1.8 s delay was used between scans. The spectra were processed with a Lorentzian-to-Gaussian filtering function and zero-filling to 2048 data points in F1, and a cosine bell squared function in F2 with zero-filling to 2048 points. Resonance intensities were used to determine the relaxation rates. ^{15}N T_2 data were analyzed using an iterative non-linear least squares determination where T_2 and M_0 were allowed to vary using the equation: $y = M_0\exp[-t/T_2]$ where y is the intensity at the relaxation time t, M_0 is the intensity when t=0, and T_2 is the ^{15}N T_2 relaxation time.

For spectra collected in chapters VI and VII: All NMR spectra were acquired at 40°C on a Varian Unity 600 NMR spectrometer. The 2D ^{1}H-^{15}N HSQC spectrum (Bodenhausen and Ruben, 1980) was acquired using 64 complex points in t_1, and 1024 complex points in t_2, with 64 scans per t_1 increment. The sweepwidths were 8000 Hz (centered at the water frequency at 4.7 ppm) in F2 (^{1}H) and 1824 Hz (centered at 119 ppm) in F1 (^{15}N). The spectrum was processed with a cosine-bell squared function in F2, and a Lorentzian-Gaussian function in F1, and both dimensions were zero filled to 2048 points. The 2D HMQC-J (Kay and Bax, 1990) spectrum was acquired with 848 complex points in

t_1, and 1024 complex points in t_2, with 128 scans per t_1 increment. The sweepwidths were 8000 Hz (centered at the water frequency at 4.7 ppm) in F2 (^{1}H) and 1824 Hz (centered at 119 ppm) in F1 (^{15}N). The spectrum was processed with a cosine-bell squared function and zero-filling to 2048 points in F2, and a series of Line broadenings together with a Gaussian function and zero-filling to 4096 points in F1. The VNMR (VNMR 4.1A, Varian, Palo Alto, CA) parameters used in F1 were either GF = 0.105, and LB = -10, -9, -8, -7, -6, -5, -4, -3, -2, and -1, or GF = 0.068, and LB = -18, -17, -16, -15, -14, -13, -12, -11, -10, -9, -8, -7, -6, -5, and -4. The splittings (in Hz) at the various LB values were fit with the program HMCQJFIT (Goodgame and Geer, 1993) which was modified for use with VNMR parameters to obtain the $^3J_{HNH\alpha}$ coupling constants. Coupling constants derived from both sets of weighting functions were within 0.5 Hz of one another indicating the coupling constants derived were accurate to approximately 0.5 Hz. For the NH exchange experiments, the ^{13}C/^{15}N TnC sample which had been lyophilized three times in D_2O at a pH of 7.6 was used. Three spectra were acquired: one three hours after final dissolution in 85% D_2O and 15% TFE and pH 7.0, one three days after dissolution, and one three weeks after dissolution. The spectra collected were ^{1}H-^{15}N HSQC experiments with sweepwidths and carrier positions as indicated above for the ^{1}H-^{15}N HSQC spectrum. The spectra were 512 (F2) x 128 (F1) complex points with 96 transients per increment. Presaturation of the small residual HOD peak was removed by application of a low power (γB_2 = 0.02 kHz) pre-saturation pulse of 1.4 s duration. The spectra were processed with a cosine bell squared function in F2 and a Lorentzian-Gaussian resolution enhancement function in F1 with zero-filling to 2048 points in both dimensions.

For backbone, sidechain, and NOE assignment of TnC, the following spectra were acquired: 3D HNCA (Grzesiek and Bax, 1992), 3D HN(CO)CA (Grzesiek and Bax, 1992), 3D HNCO (Grzesiek and Bax, 1992), 3D HCACO (Powers et al., 1991), 3D ^{15}N-edited TOCSY (75 ms mixing time) (Marion et al., 1989a), 3D ^{15}N-edited NOESY experiment (75 ms mixing time) (Kay et al., 1989), 3D HCCH-COSY (Ikura et al., 1991), 3D ^{13}C-edited NOESY (75 ms mixing time) experiment (Ikura et al., 1990), and 4D ^{13}C/^{15}N-edited NOESY (50 ms mixing time) experiment (Kay et al., 1990). All spectra were acquired with the proton carrier centered on the water frequency (4.70 ppm), and water suppression was by either a low power (γB_2 = 0.02 kHz) presaturation pulse of approximately 1.5 s duration, or by spin lock (HN(CO)CA spectrum only) water suppression (Messerle et al., 1989), or for some experiments (HCACO, HCCH-COSY, and ^{13}C-edited NOESY), no form of water suppression was needed. Sweepwidths for the proton dimension were 8000 Hz except for the HCACO experiment (3000 Hz in F3), both the HCCH-COSY and ^{13}C-edited NOESY experiments (6600 in F3 and 3600 in F1), and the ^{15}N/^{13}C-edited NOESY experiment (8000 in F4, and 4980 in F2). For those spectra edited by ^{15}N in F2 (or the ^{15}N/^{13}C-edited NOESY experiment in F3) the sweepwidth used was 1824 Hz (or 2100 Hz for the HN(CO)CA experiment) centered at 119 ppm. The sweepwidths of the ^{13}C dimension were as follows: for the HNCA, the sweepwidth was 3922 Hz with the carrier centered at 56 ppm; for the HN(CO)CA, the sweepwidth was 4500 with the carrier centered at 177.8 ppm; for the HNCO experiment, the sweepwidth was 1818 Hz, with the carrier centered at 177.8 ppm; for the HCACO experiment, the sweepwidth was 4564 Hz (F1) and 1818 Hz (F2) with the carrier centered at 56 ppm; for the HCCH-COSY, ^{13}C-edited NOESY, and ^{13}C/^{15}N-edited NOESY experiments, the sweepwidth was 4224 Hz with the carrier centered at 43 ppm. The HNCA experiment was collected with 32 (t_1) x 32 (t_2) x 512 (t_3) complex points with 192 scans per increment, the HN(CO)CA and HNCO experiments were collected with 28 (t_1) x 32 (t_2) x 512 (t_3) complex points with 192 scans per increment, the HCACO experiment was collected with 28 (t_1) x 48 (t_2) x 512 (t_3) complex points and 128 scans per increment, the HCCH-COSY was collected with 128 (t_1) x 32 (t_2) x 512 (t_3) complex points with 64 scans per increment, the ^{13}C-edited NOESY was collected with 128 (t_1) x 32 (t_2) x 512 (t_3) complex points with 32 scans per increment, the ^{15}N-edited NOESY and the ^{15}N-edited TOCSY experiments were acquired with 128 (t_1) x 32 (t_2) x 512 (t_3) complex points and either 64 scans per increment or 32 scans per increment (^{15}N-edited TOCSY), and the 4D ^{15}N/^{13}C-edited NOESY was collected with 12 (t1) x 32 (t2) x 12 (t3) x 512 (t4) complex points and 48 scans per increment. The 2D ^{1}H-^{1}H NOESY experiment was collected with 512 (t_1) x 2048 (t_2) complex points and 128 scans per increment, and the DQF-COSY experiment was collected with 512 (t_1) x 2048 (t_2) complex points and 128 scans per increment.

For the HNCA, HN(CO)CA, HNCO, and HCACO experiments, the time-domain was increased by 32 complex points in both F2 and F1 dimensions by means of linear prediction and zero-filling, so that after processing, the spectrum had a size of 1024(F3)*128(F1)*64(F2) points. The ^{15}N-edited TOCSY, and the ^{15}N-edited NOESY experiments were linear predicted in F2 only, and after processing, the final size of the spectra were 1024(F3)*256 (F1)*64(F2) points. The HCCH-COSY experiment was linear predicted in F1 only, and after processing, the final size of the spectrum was 1024(F3)*256(F1)*64(F2) points. The ^{13}C-edited NOESY experiment was linear predicted in F1, such that an additional 64 points were added, and F2, such that an additional 32 points were added, so that after zero-filling, the final size of the spectrum was 1024(F3)*512(F1)*64(F2) points. The 4D ^{13}C/^{15}N-edited NOESY experiment was linear predicted in F1, F2, and F3, such that after zero-filling the final size of the spectrum was 1024 (F4)*128(F2)*32(F1)*32(F2). The 2D ^{1}H-^{1}H NOESY experiment was zero-filled in F1 so that the final size of the spectrum was 2048(F1)* 2048(F2). The 2D DQF-COSY experiment was zero-filled in F1 so that the final size of the spectrum was 2048(F1)* 2048(F2). For all 3D and 4D experiments (except the HCACO), a post-acquisition solvent suppression by convolution of the time-domain data was applied prior to Fourier transformation (Marion et al., 1989b). All spectra were processed with either a cosine-bell squared or a 60 degree shifted sine bell squared weighting function in F1 and F2 (and F3 for the ^{13}C/^{15}N-edited NOESY experiment), and a cosine-bell squared weighting function in F3 (or F4 for the ^{13}C/^{15}N-edited NOESY experiment). After Fourier transformation of the F3 dimension, parts of the spectra without resonances were discarded, reducing the final size to between 50% and 75%. For the 2D NOESY spectrum, both dimensions were processed with a 60 degree shifted sine-bell squared weighting function, and the 2D DQF-COSY spectrum was processed with a sine-bell function in F1 and F2.

Processing of the 3D data sets was accomplished using either the VNMR software (VNMR 4.1A, Varian, Palo Alto, CA) or NMRPIPE (Delaglio et al., NIDDK, NIH, MD, unpublished). When used within VNMR software, extension of the time domain was achieved using the linear prediction algorithm lpfft. Peak-picking was carried out using the interactive graphic-based program PIPP (Garrett et al., 1991).

Assignment of backbone ^{1}H, ^{15}N and ^{13}C NMR chemical shifts: The sequential assignment of TnC was started with the HNCA experiment where the more intense ^{1}HN(i)-^{15}N(i)-^{13}Cα(i) correlation was observed. Sequential assignment was continued to the previous residue using a combination of the weaker correlation (due to the smaller two-bond coupling to the previous residue) found on the HNCA experiment coupled with the HN(CO)CA experiment at the same ^{15}N and ^{1}HN frequencies. Both the HNCA and the HN(CO)CA experiments provide the ^{1}HN(i)-^{15}N(i)-^{13}Cα(i-1) correlation. The $d_{NN}(i\pm1)$ information obtained from the ^{15}N-edited NOESY experiment, together with the ^{1}HN(i)-^{15}N(i)-^{13}C'(i-1) correlation from the HNCO experiment are also needed. The ^{13}C'(i-1) information, coupled with the ^{13}Cα(i-1) information can be used in the HCACO experiment to obtain the ^{1}Hα(i-1) correlation. The ^{1}Hα(i-1), the ^{1}HN(i±1), and the ^{13}Cα(i-1) information obtained can then be used to search for all possible ^{15}N-^{1}H amide pairs in the ^{15}N-edited TOCSY, ^{15}N-edited NOESY, and HNCA experiments that best correlate residue i to residue i-1. Because of overlap in both the ^{1}H-^{15}N dimensions, and the ^{1}Hα-^{13}Cα dimensions, the procedure frequently yielded multiple possibilities. When this was the case, the sequential assignment was continued from another place.

For spectra collected in chapter VIII: ^{1}H NMR spectra of s-TnC and the heterodimer were obtained at 600 MHz on a Varian Unity 600 NMR spectrometer. All spectra were obtained at 30 °C. Two-dimensional experiments were taken with 256 or 312 X 1024 complex data points in F1 and F2 with spectral widths of 7500 Hz. NOESY data sets were acquired using the phase-sensitive method of States et al. (1982). The appropriate phase cycling was used to achieve quadrature detection, to eliminate axial peaks, and, in the case of NOESY spectra, to eliminate multiple quantum coherences. For NOESY spectra, a random delay of between +/-10 ms was incorporated to suppress zero quantum coherences. For all data sets, the water resonance was suppressed by presaturation (1.6 - 2.3 s). The decoupler offset was set equal to the transmitter offset to prevent phase distortions around the water peak due to imperfect cancellation of the dispersive component of residual water magnetization (Hoult, 1976). For NOESY spectra, mixing times (τ_m) of 150 ms and 75 ms were used to assign resonances in the calcium-saturated s-TnC protein. For processing of 1-D spectra, a line broadening of 0.5 Hz was used. 2-D NOESY data sets were processed by applying a $\pi/8$ shifted sine bell function to the data in t_2 and a $\pi/6$ shifted sine bell function to the data in t_1 and zero-filling in t_1 to 2K data points.

III.8. Structure Calculations of TnC.

NOE-derived Distance Restraints. NOE cross peaks from the 3D ^{15}N and ^{13}C edited NOESY experiments, as well as from the 4D ^{15}N/^{13}C-edited NOESY and 2D ^{1}H-^{1}H NOESY experiments were calibrated for each residue based upon known distances. For example, for the ^{15}N-edited NOESY experiment and the ^{15}N/^{13}C-edited NOESY experiments, if the $d_{N\alpha}$ NOE could be obtained, all NOE's for that residue were classified as being stronger or weaker than that NOE. The $d_{N\alpha}$ NOE corresponds to roughly the same distance regardless of the secondary structure (2.70 to 3.05 Å). Thus, for each residue for which this NOE is found, the upper limits are calibrated according to: ucal = $3.05*(d_{N\alpha}(i)*C)^{1/6}$, where C is a measure of the accuracy of the data (a value of 3 indicates an error of 50% on the NOE) and also accounts for spin-diffusion, and the lower limits are calibrated according to lcal = $2.70*(d_{N\alpha}(i)/C)^{1/6}$. For each NOE associated with the amide of residue i, the upper bounds and lower bounds will therefore be ucal/noe$^{1/6}$, and lcal/noe$^{1/6}$ respectively. The intensity of the $d_{\alpha N}$ NOE is dependent on the type of secondary structure, but is between the broad range of 1.70 to 3.60 Å. If no $d_{N\alpha}$ NOE is observed for a particular residue, then this NOE is used for calibration of the NOE. If neither the $d_{N\alpha}$ nor the $d_{\alpha N}$ NOE's are observed, then a user-specified maximum and minimum intensity are used for the calibration. Therefore, lower bounds and upper bounds may be obtained for each NOE on a residue per residue basis. For the ^{13}C-edited NOESY and the ^{1}H-^{1}H 2D NOESY, distance restraints were scaled based upon known distances corresponding to NOE's which would be observed in these types of spectra (for example, the distance between geminal hydrogens is approximately 1.75 Å, and therefore limits of 1.8 to 2.7 Å may be used as calibration factors, between a methylene hydrogen and degenerate methyl hydrogens, limits of 2.5 to 2.6 Å may be applied, and between two methylene hydrogens on adjacent carbon atoms, limits of 2.2 to 3.1 Å may be applied). Aromatic NOE-derived distance restraints are based upon the Hγ to Hδ, and Hζ NOE of the aromatic ring (2.47 to 2.51 Å). For all cases, corrections for degenerate methylene and methyl hydrogens were automatically included to increase the upper bound in the distance restraint by 1 Å. Again, if no calibration NOE's were found for a particular residue, calibration proceeded using the maximum and minimum intensities found in the spectra. During the structure calculations, center averaging was applied to account for non-stereospecifically assigned protons (Wüthrich et al., 1983).

Torsion Angle Restraints. Torsion angle restraints for the ϕ angles were obtained as described above from an analysis of the 2D HMQC-J experiment. Only those angles for which a low error was obtained in the analysis were used as experimental restraints. For coupling constants less than 9 Hz, inclusion occurred only if a particular type of secondary structure existed as evidenced by other data such as the CSI or the $d_{N\alpha}/d_{\alpha N}$ ratio, or if during the early stages of structure calculations, the angles of an ensemble of structures tended to remain in a certain area of the Karplus curve. Most ϕ angles were given errors of $\pm 10^\circ$ except for those near the top of the Karplus curve (>9 Hz), and for those which consistently violated NOE distance restraints. ψ angles were obtained from an analysis of the $d_{N\alpha}/d_{\alpha N}$ ratio according to Gagné et al. (1994). For ratios less than 1, $\psi = 120 \pm 100$, and for ratios greater than one, $\psi = -30 \pm 110$.

Structure Calculations. Three-dimensional structures were computed from experimental restraints using a simulated annealing protocol (Nilges et al., 1988b,c) with the program XPLOR 3.1 (Brünger, 1992). The initial structure used was the model structure for calcium saturated TnC (Herzberg et al., 1986). Upon completion of the structure calculations using only simulated annealing, the TnC structure was generated from a random array of coordinates using the hybrid distance geometry / simulated annealing protocol (Nilges et al., 1988a; Kuszewski et al., 1992) to ensure no bias was placed on the final structure by the starting coordinates. The target function contained only quadratic harmonic potential terms for covalent geometry (bonds, angles, planes and chirality), square well quadratic potentials for the torsion angle restraints, a soft-square quadratic potential (simulated annealing) or square well quadratic potential (hybrid distance geometry / dynamical simulated annealing protocol) for the experimental distance restraints, and a quartic vanderWaals repulsion term for the nonbonded contacts (the XPLOR F_{repel} function was set to 0.75). Force constants for the NOE-derived distance restraints were set to 50 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{Å}^{-2}$, and the dihedral angle restraints were initialized at 5 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{rad}^{-2}$ during the high temperature dynamics and increased to 200 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{rad}^{-2}$ during the annealing stage. Annealing proceeded stepwise from 1000 K to 100 K in decrements of 50 K. The force constant on the repulsive term was increased stepwise to 4.0 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{Å}^{-4}$ and was maintained until the end of the penultimate energy minimization step which consisted of 500 cycles of Powell minimization with the vanderWaals hard-sphere radii set to 0.8 times the CHARMM values (Brooks et al., 1983). The final energy minimization step employed the 6-12 Lennard-Jones potential. The actual XPLOR input files may be found in Appendix C.

The final structure calculations were based upon 2106 NOE-determined distance restraints with 505 sequential, 321 medium-range (between 1 and 5 residues apart), and 284 long range (more than five residues apart); 121 ϕ restraints and 76 ψ restraints. The NOE and torsion angle restraint files may be found in Appendix D.

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Chapter IV:

Studies of a peptide representing calcium binding site III of TnC.

It has been shown that synthetic peptides which comprise the EF-hand helix-loop-helix calcium binding motif can undergo a calcium-induced dimerization to form homodimers (Shaw et al., 1990; Shaw et al., 1992a). The structure of these homodimers reveals symmetric two-site calcium binding domains which exhibit folds very similar to the C-terminal domain of TnC (Shaw et al., 1992b; Kay et al, 1991). The use of such small synthetic peptides as probes in understanding muscle structure and function has been widespread (Van Eyk and Hodges, 1993).

Troponin C isolated from turkey, chicken or rabbit has a single cysteine residue at position 101 which is located in the N-terminal helical arm of calcium binding site III. Previous studies of SCIII peptides (Shaw et al., 1990, 1991, 1992), have involved synthesizing an alanine in place of the cysteine. An alternative to mutation of the cysteine to an alanine, is alkylation of the peptide with a sulfhydryl reagent. The sulfhydryl reagent could have a reporter group located on it (for example a fluorine group) which could monitor calcium binding (using ^{19}F NMR). The cysteine in TnC has been exploited in many studies over the years where it has been alkylated with various spectroscopic probes in order to monitor calcium binding to TnC (Ohnishi et al., 1975; Potter et al., 1976; Grabarek et al., 1983; Wang and Cheung, 1985). As well, it has been altered to study the complexation of TnC with other members of the troponin complex either through observing changes in fluorescence (Wang and Cheung, 1984, 1985, Cheung et al., 1991), NMR spectra of paramagnetic spin labels (Dalgarno et al., 1982), or crosslinking (Chong and Hodges, 1981, 1982; Tao et al., 1986, 1989; Leszyk et al., 1987, 1988). It is therefore extremely important to understand if there are significant changes upon alkylation of this residue, and if these changes affect calcium binding to, or the structure of, the protein. Due to its small size, relative ease of study, and the fact that NMR information on the SCIII homodimer was available, it was chosen as a model for determining the effect of alkylation. SCIII was synthesized with a cysteine at position 101 and alkylated with the sulfhydryl reagent: 3-bromo-1,1,1-trifluoropropanone. This alkylating reagent efficiently and preferentially labels cysteine residues. Presented here is a study of the calcium binding properties and secondary structure of SCIII labeled at cysteine 101 with the sulfhydryl reagent 3-bromo-1,1,1-trifluoropropanone (S-TFP-C101-SCIII). Details of the synthesis and alkylation procedure of SCIII may be found in Chapter III.

Sequence-Specific Assignment. The assignment of calcium-saturated S-TFP-C101-SCIII was carried out through the identification of various spin systems in 1D, DQF-COSY, TOCSY and NOESY spectra. Using these experiments, the spin systems of the tyrosine (112), two phenylalanine (102, and 105), four isoleucine (104, 113, 115, and 121), three glycine (111, 119, and 126), and three alanine (99, 109, and 124) residues were identified. As well, the N-terminal residue (lysine 93) was identified from a strong NOE between the amide proton and the N-terminal acetyl group. Additionally, assignments were made for some AMX spin systems which could not be identified a-priori (three aspartic acid residues (106, 110, and 114), two asparagine residues (100, and 108), one serine residue (94), and one cysteine residue (101)). The remaining residues

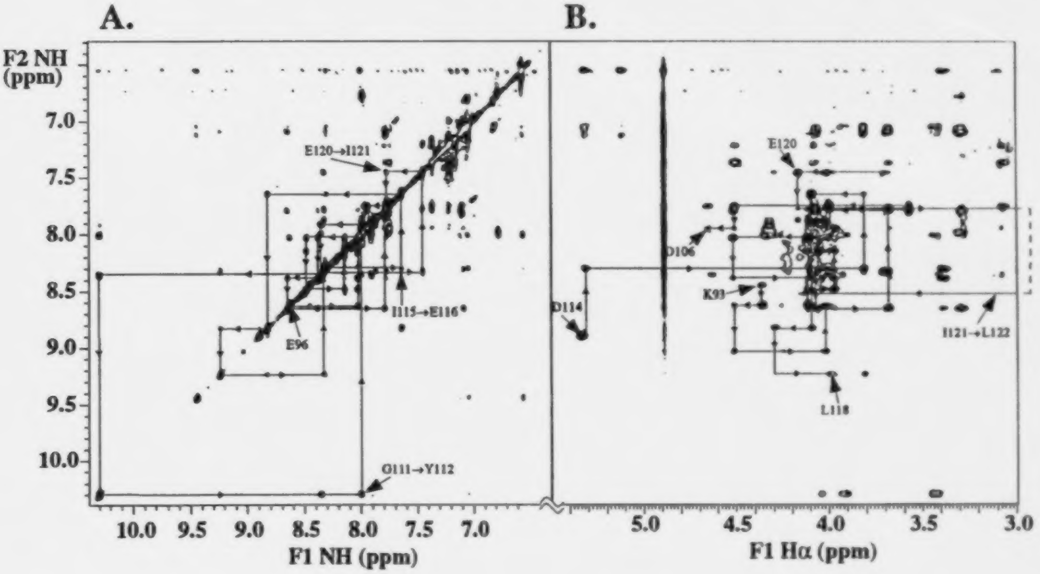

Figure IV.1 500 MHz ^1H-^1H 2D-NOESY spectrum of calcium-saturated 3.1 mM S-TFP-C101-SCIII showing (A) NH-NH connectivities and (B) NH-Hα connectivities. Conditions for the experiments are described in Chapter III.

(lysine 107, glutamic acids 95, 96, 97, 116, 117, 120, leucines 98, 118, and 122, arginines 103, and 123, and threonine 125) were identified after sequential assignment was made.

Figure IV.1A illustrates the NH-NH region of a NOESY spectrum where the d_{NN} connectivities are shown, and Figure IV.1B illustrates the NH-Hα region of the NOESY spectrum showing the $d_{\alpha N}$ connectivities. The complete list of assignments for S-TFP-C101-SCIII is given in Table IV.1.

Table IV.1 ^{1}H Chemical shifts for S-TFP-C101-SCIII

	Chemical Shift (ppm)				
Residue	**NH**	**Hα**	**Hβ**	**Hγ**	**Others**
Acetyl					Me = 2.03
K93	8.44	4.36	1.73, 1.44	1.80, 1.80	δ, δ' = 1.64, ε, ε' = 2.96
S94	8.62	4.51	4.24, 3.98		
E95	9.03	4.02	2.05, 1.78	2.28*, 2.28*	
E96	8.63	4.11	2.19, 2.02	2.43, 2.40	
E97	8.02	4.11	2.29, 2.04	2.35, 2.35	
L98	8.48	3.97	1.78, 1.60	1.72	δ = 0.86, δ' = 0.72
A99	8.14	4.05	1.46		
N100	8.02	4.51	2.89, 2.89		
C101	8.38	4.07	3.38, 2.75		TFP (CH_2) = 2.03
F102	8.65	3.68	3.28, 2.95		δ, δ' = 7.12, ε, ε' = 7.03, ζ = 6.80
R103	7.79	4.06	2.09, 2.09	1.80, 1.65	δ, δ' = 3.27, 3.19, NH = 7.30
I104	7.78	3.56	1.54	1.49, 0.98	γCH_3 = 0.26, δCH_3 = 0.66
F105	7.74	4.50	3.06, 2.58		δ, δ' = 7.36, ε, ε' = 7.21, ζ = 7.14
D106	7.94	4.65	2.76, 1.91		
K107	7.86	4.10	1.93, 1.32*	1.57, 1.57	δ, δ' = 1.80, ε, ε' = 3.06
N108	7.97	4.75	3.28, 2.84		
A109	7.91	4.03	1.35		
D110	8.35	4.62	3.08, 2.43		
G111	10.29	3.91, 3.42			
Y112	8.00	5.11	2.76, 2.47		δ, δ' = 6.54, ε, ε' = 6.54
I113	9.44	5.34	2.24	1.01, 0.84	γCH_3 = 0.89, δCH_3 = 0.32
D114	8.89	5.31	3.37, 2.84		
I115	8.29	3.81	0.96	1.14, 0.98	γCH_3 = 0.74, δCH_3 = 0.59
E116	7.64	4.09	2.15, 2.53*	2.41*, 2.26*	
E117	8.82	4.29	2.54, 2.16	2.92, 2.45	
L118	9.23	3.98	2.26, 1.48	1.64	δ = 1.05, δ' = 0.81
G119	8.32	4.02, 3.69			
E120	7.45	4.16	2.39, 2.27	2.35*, 2.04	
I121	7.77	2.83	1.76	1.29, -0.41	γCH_3 = 0.38, δCH_3 = 0.52
L122	8.52	4.15	1.99, 1.99	1.60	δ = 0.91, δ' = 0.91
R123	7.74	3.99	2.07, 1.95	1.81, 1.57	δ, δ' = 3.22, NH = 7.51
A124	7.91	4.32	1.60		
T125	8.00	4.35	4.28		γCH_3 = 1.21
G126	7.95	3.96, 3.89			
Amide					

Chemical shifts are referenced to the methyl resonances of DSS at 0.0 ppm. All data was recorded at 15° C. Those assignments marked with a '*' are tentative.

Secondary Structure. Upon the complete assignment of S-TFP-C101-SCIII, the secondary structure was determined based upon NOE connectivities, the chemical shift index, and the ratio of the $d_{N\alpha}/d_{\alpha N}$ NOE's. As shown in Figure IV.2, two regions of α-helical structure were found where contiguous stretches of $d_{\alpha N}(i,i+3)$, and $d_{\alpha\beta}(i,i+3)$ connectivities were observed. Consistent with these data is the chemical shift index for the α-protons (Wishart et al., 1991), the presence of strong d_{NN} NOE's which indicate helical structure, and $d_{N\alpha}/d_{\alpha N}$ ratios (which are greater than one) indicative of helical secondary structure. The two helices appear to encompass residues 95 to 105, and 115 to 124. The residues between these two helices (106 to 114), however, show significantly different NOE's. d_{NN} connectivities were found for residues 106 to 111, but no $d_{\alpha N}(i,i+3)$ or $d_{\alpha\beta}(i,i+3)$ NOE's were found. The $d_{N\alpha}/d_{\alpha N}$ ratios in this region are not regular (some are ambiguous, one is less than one, and some ratios are equal to 1 or are not observed), suggesting that perhaps there is no regular secondary structure. Residues 112, 113, and 114 appear to be in an extended conformation as evidenced by the chemical shift index, and no d_{NN} connectivities.

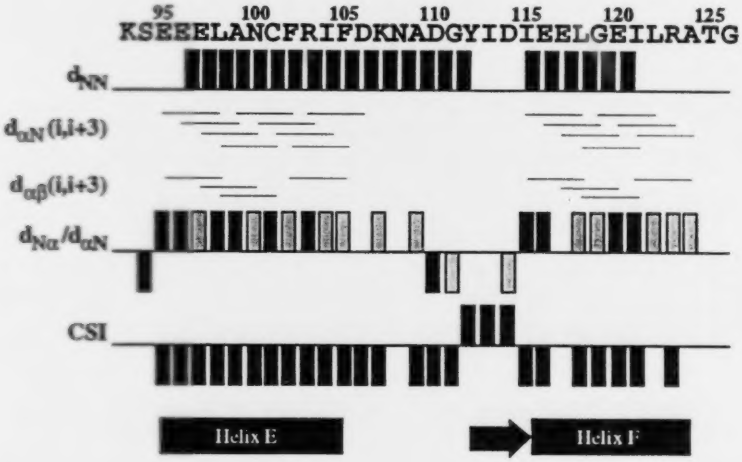

Figure IV.2. Summary of the secondary structure of S-TFP-C101-SCIII illustrating sequential and medium range NOEs involving amide protons, the ratio of the $d_{N\alpha}/d_{\alpha N}$ intensities, and the chemical shift index (for α-protons only). For the $d_{N\alpha}/d_{\alpha N}$ ratio, the boxes pointing up represent a ratio >1 whereas the boxes pointing down represent a ratio <1. Gray boxes represent ratios for which either the $d_{N\alpha}$ or $d_{\alpha N}$ NOE's are missing and it is assumed that the intensities are small. No box represents either a ratio of ~ 1 or both the $d_{N\alpha}$ and $d_{\alpha N}$ NOE's are ambiguous. For the CSI (chemical shift index), the boxes pointing up represent β-sheet secondary structure, and the boxes pointing down represent α-helix secondary structure. No box indicates a random coil chemical shift for the Hα. The secondary structure elements are marked according the criteria shown where boxes represent α-helix and arrows represent β-sheet. Residues and their numbering are indicated above.

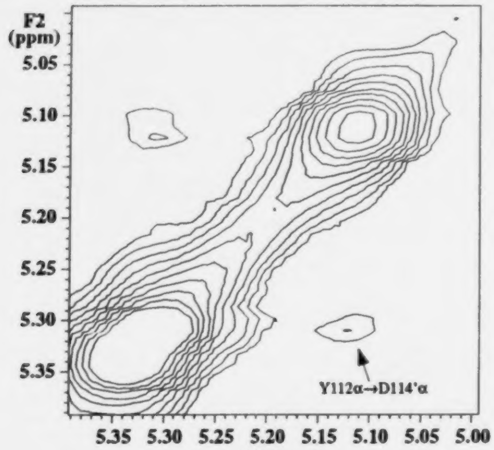

Figure IV.3 500-MHz 2D ^{1}H-^{1}H NOESY spectrum in D_2O at 15°C of 3.1 mM S-TFP-C101-SCIII illustrating the Hα-Hα region. Shown is the NOE connectivity for Y112 to D114.

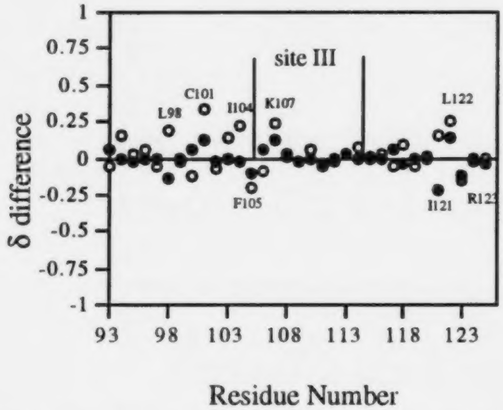

Figure IV.4. Difference in backbone NMR chemical shift data between S-TFP-C101-SCIII (at 15°C) and SCIII (at 30°C) homodimers. Shown are the NH (open circles) and Hα (closed circles) shift differences. Shifts for the SCIII homodimer were taken from Shaw et al.(1992b).

In the paper of the tertiary structure determination of SCIII (Shaw et al., 1992b), it was shown that there were several NOE contacts between helices E and F (where helix E is the first helix and helix F is the second helix). Upon comparison with the x-ray structure of the C-terminal domain of TnC (Herzberg and James, 1988; Satyshur et al., 1988), these NOE's would only make sense if two helix-loop-helix monomers were to associate to form a symmetric dimer (Shaw et al., 1990). The essential requirement of the SCIII dimer would be a short three-residue β-sheet involving residues tyrosine 112, isoleucine 113, and aspartic acid 114 of each peptide which would correspond to residues 112 - 114 and 148 - 150 of site III and site IV of the crystal structures (Herzberg and James, 1988; Satyshur et al., 1988) (Shaw et al., 1992b). Figure IV.3 illustrates a NOESY spectrum in D_2O where an NOE is shown between Y112 Hα to D114 Hα which could only occur in the extended structure if there was a β-sheet dimer interface.

The results shown here are consistent with those of Shaw et al (1990, 1992b). Two regions of helical structure are present in each monomer, and a β-sheet structure links two SCIII monomers together. Further analysis of the NOESY spectra reveal several interhelical contacts between monomers which could only be assigned as intermonomer NOE's, also consistent with the results of Shaw et al. (1992b) (data not shown).

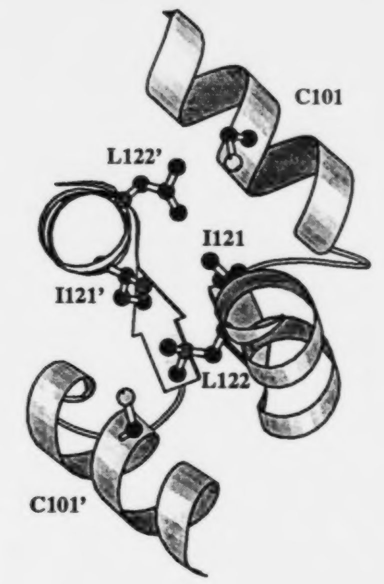

Figure IV.5. Average energy minimized NMR-derived structure of the SCIII homodimer (Shaw et al., 1992b), where alanine 101 of SCIII has been mutated to a cysteine to illustrate the proximity of the sidechains of cysteine 101, isoleucine 121, and leucine 122,. This figure was generated using MOLSCRIPT (Kraulis, 1991).

Figure IV.4 graphically illustrates the difference in chemical shift of the NH protons and the Hα protons between SCIII and S-TFP-C101-SCIII. In general, the chemical shift differences are not large between the two proteins considering that both were assigned at different temperatures. The residues which show the largest chemical shift differences are L98, C101, I104, and F105 in the first helix, as well as K107 in the calcium binding loop region, followed by I121, L122, and R123 in the second helix. The alteration of the chemical shift of residues on the same helix as S-TFP-C101 is to be expected, since the α-helix has 3.6 residues per turn which implies that every third to fourth residue will be affected to some degree by C101. Assuming that the structures of both SCIII and S-TFP-C101-SCIII are the same, Figure IV.5 illustrates how residues I121, and L122 would be oriented with respect to C101. This figure shows that the sidechain of C101 points into a pocket formed by I121 of the same monomer and L122 of the symmetrically related monomer. The chemical shift difference of these residues is therefore most likely due to the effect of the TFP group. The data is supportive of a symmetric homodimer forming since the sidechain of C101 appears to make contacts with L122 on the symmetrically related monomer.

Stoichiometry of calcium binding to S-TFP-C101-SCIII. In the absence of added calcium, S-TFP-C101-SCIII appears unstructured as the ^{1}H spectrum of the apo peptide exhibits relatively narrow lines. As was shown for SCIII (Shaw et al., 1991a), most of the chemical shifts of the apo peptide fall within the range of random coil peptides. The addition of calcium introduces dramatic changes within the spectrum. Figures IV.6 and IV.7 illustrate ^{1}H, and ^{19}F 1D NMR spectra at different calcium:peptide ratios. It is observed in both types of spectra that as calcium is added, new resonances appear and increase in intensity consistent with slow exchange kinetics. Figure IV.6 illustrates the change in chemical shift of the Y112 δ and ε protons. In the apo peptide, these resonances are a pair of doublets resonating at approximately 7.1 ppm and 6.8 ppm respectively. Addition of calcium causes these resonances to shift to higher field and eventually appear almost degenerate at 6.6 ppm. At this point, it should be noted that there is a resonance (F102 ζH) at 6.8 ppm which can clearly be seen in the calcium-saturated peptide. This resonance is also present in the apo peptide but is obscured by the resonances of the Y112 ε doublet.

The binding of calcium was quantitated by plotting the calcium:peptide ratio versus the integral of the Y112 resonances appearing at 6.6 ppm (Figure IV.8). The stoichiometry was determined from an intersection of the initial slope at low calcium:peptide ratios and the slope at maximum intensity. The two lines intersected at a calcium:peptide ratio of approximately 0.60. This value is almost identical to that obtained by Shaw et al. (1991a) (0.57).

A stoichiometry of approximately 0.5 calcium:peptide suggests that calcium binds to one unstructured peptide and induces a conformational change to form a helix-loop-helix peptide. This peptide associates with an apo-SCIII molecule, causing it to undergo an identical conformational change forming a dimer with one calcium molecule. This dimer is then free to bind a second equivalent of calcium. The fact that only one set of

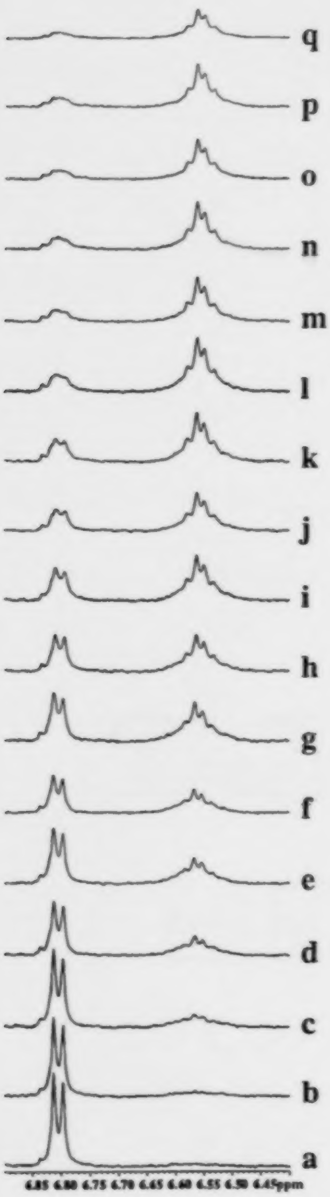

Figure IV.6. 500 MHz ^{1}H NMR spectra of 492 μM S-TFP-C101-SCIII at the following calcium:peptide ratios: (a) 0.012, (b) 0.057, (c) 0.148, (d) 0.240, (e) 0.284, (f) 0.330, (g) 0.376, (h) 0.420, (i), 0.466, (j) 0.510, (k) 0.553, (l) 0.647, (m) 0.737, (n) 0.829, (o) 0.921, (p) 1.012, (q) 1.191. Conditions of the experiment are as described in Chapter III.

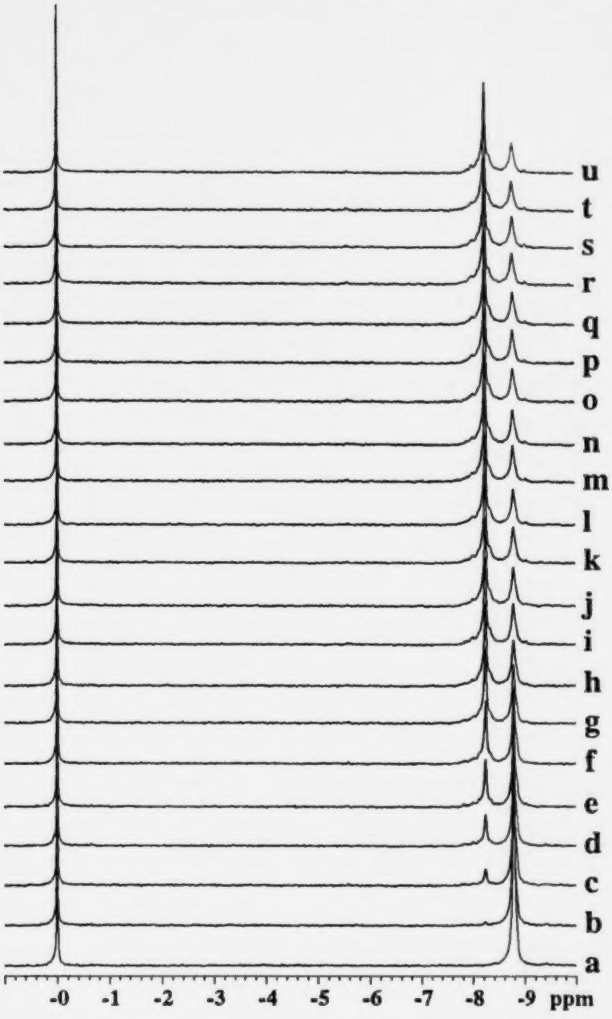

Figure IV.7 282-MHz ^{19}F NMR spectra of 459 μM S-TFP-C101-SCIII at the following calcium:peptide ratios: (a) 0, (b) 0.05, (c) 0.14, (d) 0.24, (e) 0.34, (f) 0.43, (g) 0.53, (h) 0.62, (i) 0.72, (j) 0.81, (k) 0.91, (l) 1.00, (m) 1.10, (n) 1.29, (o) 1.48, (p) 1.68, (q) 2.16, (r) 2.63, (s) 3.59, (t) 4.55, (u) 5.51. Conditions are as described in Chapter III.

resonances could be assigned in the calcium saturated state illustrates that each of the subunits of the S-TFP-C101-SCIII dimer has the same conformation.

Figure IV.7 illustrates the binding of calcium by S-TFP-C101-SCIII using ^{19}F NMR as a monitor of calcium binding. As for the ^{1}H NMR calcium titration, the addition of calcium causes a change in the chemical shift of the ^{19}F resonance consistent with slow exchange kinetics.

A plot if the integrated peak area versus the calcium:peptide ratio (Figure IV.9A) illustrates that the stoichiometry of calcium binding to the peptide is virtually identical to that obtained using ^{1}H NMR methods. The ^{19}F NMR data shown in Figure IV.7, however, are different from the ^{1}H NMR data. In Figure IV.7, there are two upfield shifts (relative to TFA), one at -8.8 ppm which corresponds to S-TFP-C101-SCIII with no calcium bound, and one at -8.2 ppm which corresponds to S-TFP-C101-SCIII with calcium bound. As the calcium concentration is increased, the peak at -8.8 ppm decreases in intensity, but does not vanish even up to calcium:peptide ratios of 5.5:1. At a calcium:peptide stoichiometry of 0.66, the titration does not appear to change (Figure IV.9A). An analysis of the linewidth of the two peaks as a function of the calcium:peptide ratio (Figure IV.9B) reveals that the linewidth of the calcium-bound species remains constant throughout the titration (the peak at -8.2 ppm), whereas the linewidth of the calcium-free species (peak at -8.8 ppm) titrates with calcium, reaching a maximum at the stoichiometry of calcium binding (0.66). The linewidth of the free peak (calcium-saturated) is almost twice the linewidth of the bound peak suggesting that some sort of exchange broadening is occurring. It therefore follows, that while ^{1}H NMR monitors the conformation of the peptide, ^{19}F NMR monitors calcium binding to the peptide. An analysis of the integration of the two fluorine peaks of fully calcium saturated S-TFP-C101-SCIII (calcium:peptide > 5) reveals that approximately 70% of the peptide is in the calcium saturated form, and 30% is calcium free. Since no other species is present in solution (as evidenced by only one set of ^{1}H shifts), this data confirms the hypothesis of an asymmetric dimer in terms of calcium binding. The fact that the data indicates calcium binds at a slightly higher calcium:peptide ratio than 0.5:1 cannot be explained at this time. The slightly higher ratio could be due to experimental errors, or perhaps the calcium concentration was not increased high enough to show metal binding to the second calcium binding site.

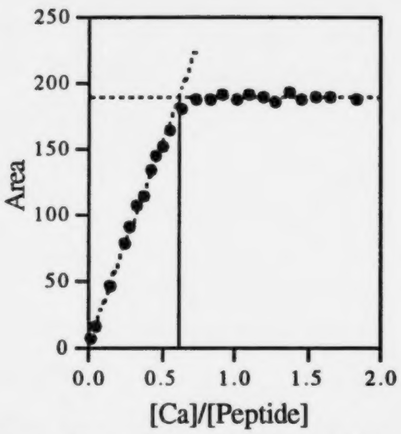

Figure IV.8. Calcium titration plot for the S-TFP-C101-SCIII titration shown in Figure IV.5. The increase in volume of the Y112 peak at 6.6 ppm was measured as a function of the calcium:peptide ratio. The two dashed lines indicate the slopes for the data at low and high calcium:peptide ratios. The vertical solid line is the intersection of the two lines.

The secondary structure and calcium binding properties of S-TFP-C101-SCIII appear to be virtually identical with the results of Shaw et al. (1992b, 1991b) on SCIII which has an alanine residue in place of the cysteine. S-TFP-C101-SCIII forms a symmetric homodimer upon binding calcium with a stoichiometry of calcium binding of approximately 0.63 calcium:peptide. These results suggest that the use of small alkylating reagents for modification of cysteine 101 of chicken or turkey skeletal TnC or cysteine 98 of rabbit TnC does not have a major effect on the calcium-binding properties nor on the structure. The studies of TnC in the later chapters in this thesis involve the use of the alkylating reagent iodoacetamide for modification of cysteine 101. The results presented here illustrate that carboxamidomethylated TnC should not have any major structural defects due to the presence of the carboxamidomethyl group, and therefore should have the same properties as native TnC.

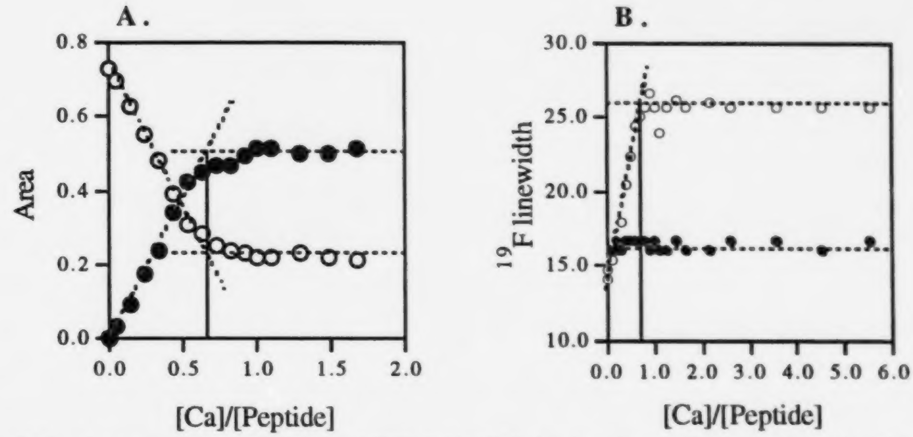

Figure IV.9. Calcium titration plot for the S-TFP-C101-SCIII titration shown in Figure IV.7. (A) Plot of the area under the peak versus the calcium:peptide ratio. The lines are as indicated in Figure IV.8. (B) Plot of the linewidth of the free peak (-8.8 ppm) and the bound peak (-8.2 ppm) as a function of the calcium:peptide ratio.

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Chapter V:

The calcium-induced dimerization of troponin C: the mode of interaction and the use of trifluoroethanol as a denaturant of quaternary structure.[1]

Troponin C (TnC) is the calcium-binding protein of the thin filament of muscle involved in the regulation of muscle contraction. Chicken skeletal TnC binds four metal ions in four separate metal ion binding sites numbered consecutively from the N-terminus of the protein. The structure of half-saturated avian skeletal TnC (with calcium binding sites III and IV filled) has been solved using x-ray crystallographic techniques (Satyshur et al., 1988, 1994; Herzberg and James, 1988) and reveals two domains (the N-terminal and the C-terminal domains), each with two EF-hand helix-loop-helix calcium binding sites. The N-terminal domain contains the lower affinity regulatory calcium specific sites whereas the C-terminal domain contains the high affinity calcium/magnesium binding sites. Knowledge of the structure of TnC in its half and fully calcium saturated forms is essential to understanding the molecular mechanism of muscle contraction.

Nuclear magnetic resonance (NMR) spectroscopy has developed into a technique capable of determining three dimensional structures of proteins in solution. Developments in molecular biology have allowed not only large amounts of proteins to be expressed, but have facilitated the incorporation of ^{15}N and ^{13}C isotope labels into proteins. Spectral editing and internuclear correlations based on the attached heteronuclei coupled with greater dimensionality, result in better resolution than possible by two-dimensional methods. Our goal has been to use these new techniques to solve the 3D solution structure of calcium saturated TnC. TnC, however, undergoes a reversible calcium-induced dimerization. This dimerization/aggregation is detrimental to the NMR structural characterization of TnC since the molecular weight of the dimer is beyond the limits of present NMR techniques.

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[1] A version of this chapter has been published. Slupsky, C.M., Kay, C.M., Reinach, F.C., Smillie, L.B., & Sykes, B.D. 1995. Biochemistry 34: 7365-7375.

The first characterization of the calcium-induced dimerization of TnC involved hydrodynamic studies using ultracentrifugation techniques (Murray and Kay, 1972). The authors showed that TnC aggregated at saturating calcium concentrations and neutral pH. It was later shown that the calcium-induced dimer had a K_d of 0.08 mM (at pH 7.0, and 5° C in 10 mM imidazole, 5 mM potassium phosphate, 1 mM $MgCl_2$, and 1 mM DTT), and a monomer apparent molecular weight of 18 700 was estimated from a two-species plot of the data (Margossian and Stafford, 1982). TnC aggregation has also been studied using small angle x-ray scattering techniques (Fujisawa et al., 1990; Blechner et al., 1992).

In addition to the calcium-induced dimerization, TnC also undergoes a reversible acid-induced aggregation (Wang et al., 1989). As the pH of TnC is lowered from 7 to 5, the affinity of the N-domain sites for calcium is reduced (McCubbin et. al., 1986). The crystal structure of TnC was determined at a pH near 5, where the N-domain sites are in the apo form. The acid-induced aggregation is most likely similar to the molecular packing of TnC seen in the crystal which involves helix A of the N-domain interacting with the C-domain hydrophobic pocket (Satyshur et al., 1988; Herzberg and James, 1988). The interaction is hydrophobic in nature and allows for polymerization of TnC. This paper will show that the mode of the calcium-induced dimerization is different from the acid-induced aggregation.

Protein aggregation can be a general problem especially as a large number of proteins become available for structural studies at fairly high concentrations using solution techniques such as NMR spectroscopy. Some proteins are completely insoluble in water, such as membrane proteins (Barsukov et al., 1990; Orekhov et al., 1994; Johansson et al., 1994; Girvin and Fillingame, 1993; Lycksell et al., 1992), whereas others are "sticky" and thus aggregate at higher concentrations. It has recently been shown that one way to prevent protein aggregation during NMR studies has been to use the detergent CHAPS (Anglister et al., 1993; Grzesiek and Bax, 1993; Davies and Riechmann, 1994). Other methods used to break up protein aggregation have included the use of organic cosolvents. One of the more studied protein aggregates is insulin. A variety of cosolvents have been used to overcome its self-association, such as 35% acetonitrile (Kline and Justice, 1990), 20% acetic acid (Hua and Weiss, 1991a), 10% DMSO (Hua and Weiss, 1991b) or 33% trifluoroethanol (Higgens et al., 1990; Craik and Higgens, 1991). We have chosen to use the solvent trifluoroethanol (TFE) to prevent TnC aggregation.

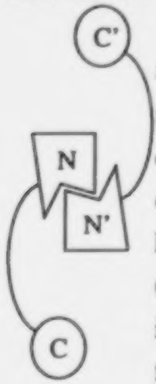
TFE has been characterized as a structure-enhancing cosolvent. TFE has been shown to stabilize a variety of structures in peptides including β-sheet (Goodman et al., 1971; Mutter and Altmann, 1985; Maser et al., 1984), β-turns (Cann et al., 1987; Greff et al., 1976; Siligardi et al., 1987; Blanco et al., 1994) and α-helix (Nelson and Kallenbach, 1989; Lu et al., 1984; Lehrman et al., 1990; Segawa et al., 1991; Marion et al., 1988; Jiméniz et al., 1987; and Reutimann et al., 1981). The dielectric constant of TFE is approximately one third that of water, and it was proposed that the net effect of TFE would be to strengthen the interactions between charged groups (Llinás and Klein, 1975; Nelson and Kallenbach, 1986). It was shown, however, that secondary structure is stabilized due to the decreased hydrogen bonding of amide protons to the solvent resulting in increased intramolecular hydrogen bonds (Nelson and Kallenbach, 1986; Sönnichsen et al., 1992). TFE has also been found to disrupt hydrophobic protein interactions without substantial structural change of the individual monomeric units involved, thereby acting as a denaturant of quaternary structure (Lau et al., 1984 a,b). The fully folded native structure of hen egg white lysozyme was studied with 15% v/v TFE, and it was shown that the structure of this protein was relatively unchanged at this low concentration of TFE (Buck et al., 1993). This paper also illustrates that for this concentration of TFE, no substantial structural changes occur for TnC.

The present investigation indicates a calcium-induced dimerization of TnC, using ultracentrifugation and NMR studies, and shows that this dimerization involves the respective N-domains of the two monomeric units. Further, this interaction is hydrophobic in nature and may be disturbed upon addition of a small amount of TFE. TFE does not significantly alter the characteristics of TnC as a calcium binding protein nor does it alter the tertiary structure of the monomer. The added TFE thereby permits NMR structural studies to be accomplished on this protein. Implications involving the use of TFE as a general perturbant of quaternary structure are also discussed.

Results:

Troponin C has been shown in the crystal structure to be a dumbbell shaped molecule with separate N- and C-terminal domains attached by a helical linker (Herzberg and James, 1988; Satyshur et al., 1988, 1994). The first indication that the calcium induced dimerization of TnC in solution involves the interaction of the N-terminal domain from two monomeric TnC units was obtained from the observation of differential linewidths of NMR resonances from the N- and C- terminal domains, and the effect of temperature on this phenomenon. In 1D NMR spectroscopy, the peak height increases as the linewidth decreases; in 2D NMR, the crosspeak intensity reflects the linewidths of the two correlated resonances because of relaxation in the indirectly detected dimension and line-broadening in the observed dimension. Since the linewidths in NMR spectra increase with increasing molecular weight, the cross-peak intensity in nD spectra are therefore increasingly sensitive to larger molecular weights (Weiss et al., 1984). Figure V.1 presents a study of the effect of temperature on cross peak intensity in 2D NMR spectra of calcium-saturated TnC. Shown are 2D ^{1}H - ^{15}N HSQC NMR spectra correlating the proton and nitrogen chemical shifts of the amide residues of TnC. At 10°C, the most obvious feature of this spectrum is the differential intensities of crosspeaks from the N- and C- domains, with some N-domain crosspeaks not even visible. The residues T39, G43, G50, and T54 are broadened to the point where they are not visible. The other N-domain peaks (D32, G33, G34, G69, and T72) are also very broad and are barely visible. The C-domain crosspeaks, however, are all present, and all have strong intensities in comparison to the N-domain peaks. As the temperature is increased, the intensity of the N-domain cross-peaks (D32, G33, G34, G43, G50, T54, V65, G69, and T72) gradually increase, whereas the intensity of the C-domain cross-peaks (G119, T125, G126, S141, and G160) remain fairly constant. At much higher temperatures, however, some cross-peaks in the ^{1}H-^{15}N HSQC NMR spectrum are lost due to increased amide exchange with the solvent; see for example the residues threonine 39 and glycine 92 at 50°C.

There is a difference in the molecular weight of the N-terminal (90 residues) and C-terminal (72 residues) domains. This difference, however, is not sufficient to explain the difference in intensities nor the change in intensities as the temperature is increased, as this change should be the same for both domains regardless of their size. Further, any difference between the two domains could only be seen if there was a flexible linker between them, or if the internuclear vectors dominating the relaxation of the observed nuclei all had a different orientation relative to the principal axis system of an anisotropic diffusion tensor for a rigid dumbbell molecule (Barbato et al., 1992). A simple model consistent with these data is therefore proposed wherein the dimerization involves the association of two N-terminal domains to which the C-terminal domains are attached via a flexible linker as shown below. As the temperature is raised, not only are the linewidths of resonances from both the N- and C-terminal domains decreased slightly (or intensities increased slightly) by viscosity and temperature effects, but the monomer-dimer equilibrium is shifted towards the monomer resulting in an additional strong decrease in linewidth for resonances from the N-terminal domain.



Scheme V.1

It was previously shown for the homologous and also dumbbell shaped protein calmodulin, that both domains have different average ^{15}N-T_2 relaxation rates and thus different average correlation times, and that the correlation times derived for the individual amides depend only weakly on the orientation of the N-H bond vector (Barbato et al., 1992). Thus, it was hypothesized that the central helix of calmodulin serves as a flexible tether (Barbato et. al., 1992). There is no evidence, however, for a calcium induced dimerization of calmodulin. The difference in linewidths observed for the two domains of calmodulin is much less than observed here and similar to that observed for TnC in TFE (see below).

Figure V.2A represents low speed sedimentation equilibrium studies on TnC in the presence of calcium and either the presence or absence of TFE. These data were fit to a monomer and dimer equilibrium (see Chapter III). The molecular weight that best fits the ultracentrifugation data at 20°C is 18 415 for both TnC and TnC in TFE which is close to the calculated molecular weight of 18 315 daltons. The K_d for TnC at 20°C was determined to be 0.43 mM in the absence of TFE, and 4.2 mM in the presence of TFE. If one assumes that the K_d changes by a factor of approximately 2 for every 10°C, then our results would predict a K_d of 0.14 mM at 5°C which is within experimental error of that obtained by Margossian and Stafford (1982). It is thus possible to estimate a K_d of 1.6 mM for TnC and 16 mM for TnC in TFE at 40°C (assuming that there is a 10 fold increase in K_d in the presence of TFE at all temperatures). Figure V.2B is a plot of the percent of monomer or dimer versus the log of the total concentration of TnC at 40°C. This plot indicates that in the absence of TFE, the concentration of TnC where half is monomer and half is dimer is 1.5 mM, whereas in the presence of TFE, the concentration of TnC where half is monomer is approximately 15 mM. For 1.4 mM TnC in the presence of TFE (the concentration used in our studies), the amount of monomer present is at least 87%.

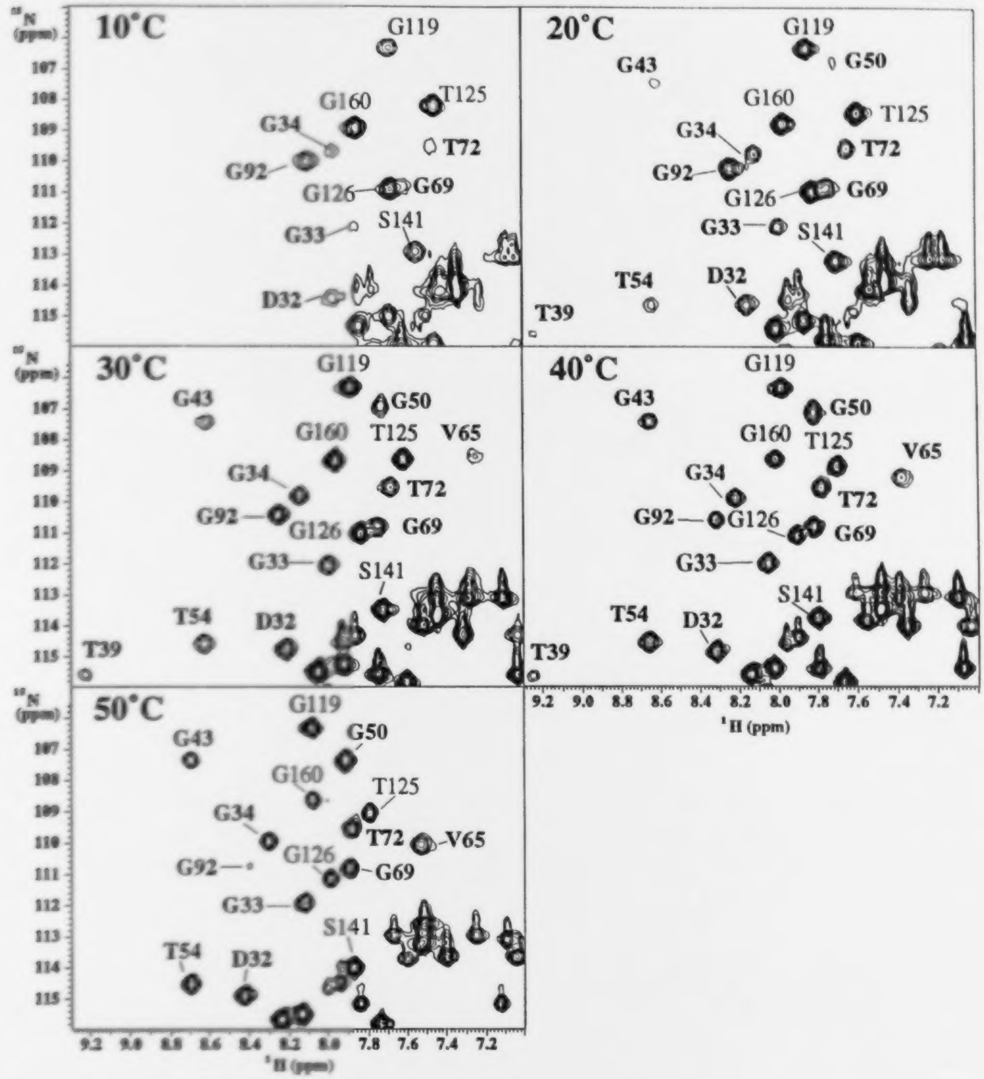


Figure V.1: A study of the effect of temperature on the intensity of crosspeaks in a series of 600 MHz 2D ^{1}H-^{15}N HSQC NMR spectra recorded with spin-lock water suppression (Messerle et al., 1989) of TnC which has been uniformly labeled with ^{15}N (>95%). Shown is the portion of the HSQC spectrum corresponding to glycine residues. Crosspeaks labeled in bold correspond to resonances from the N-domain, while those that are not correspond to resonances from the C-domain (for assignments, see Chapter VI).

Figure V.3 represents an NMR monitored titration of TnC with TFE. Shown are amide correlations in the downfield region of 2D ^{1}H-^{15}N HSQC NMR spectra. Again, one can see in the panel for 0% TFE the differential intensities for the crosspeaks for the residues from the N- and C-terminal domains. I37, I73, and D74 are barely visible, and

K23 is weaker in intensity at 0% TFE. As the amount of TFE is increased, the intensities of crosspeaks arising from the N-domain (K23, I37, D74, and I73) increase whereas the intensities of crosspeaks arising from the C-domain (I113, D114, E131, I149, D150, and F154) remain virtually constant. These differences may be seen more quantitatively in Figures 4A and 4B where residues in homologous positions in both the N and C domains are compared; I37 in the β-sheet portion of calcium binding site I, and I113 in the β-sheet portion of calcium binding site III. The intensity and ^{1}H linewidth of I113 remains constant throughout the TFE titration whereas the intensity and ^{1}H linewidth of I37 increases or decreases, respectively to a level comparable to I113. This plot shows that the minimum concentration of TFE that could be used, so that both N and C domain crosspeaks were similar in intensity and linewidth, was 15% v/v at 40°C. The maximum attainable concentration of TnC at 15% v/v TFE was 1.4 mM.

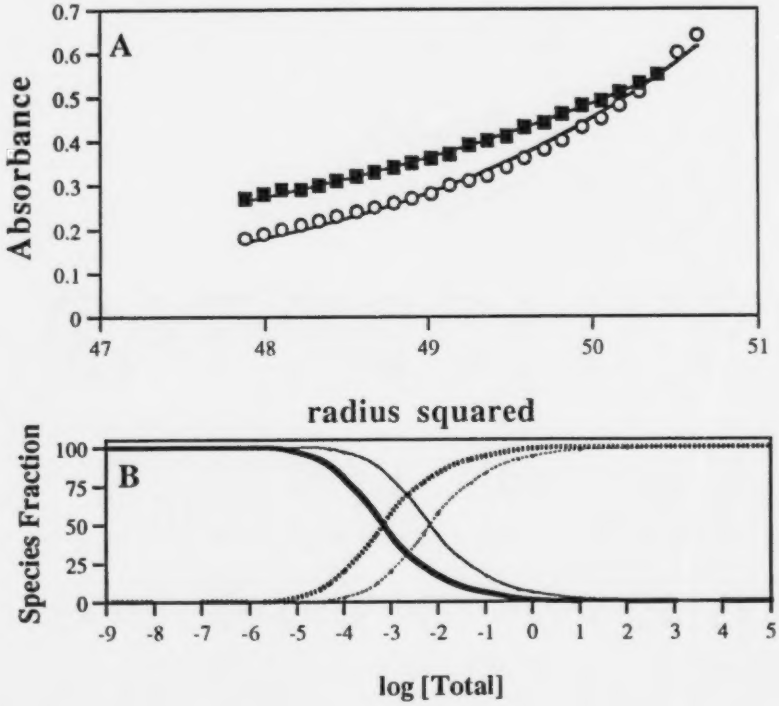


Figure V.2: (A) Sedimentation equilibrium data for TnC in the presence (indicated by the filled squares) and absence (indicated by the open circles) of TFE. Shown is the absorbance (1cm path length) versus the radial distance (to the center of the rotor) squared. The data was fit as outlined in Chapter III, and molecular weights and dissociation constants were obtained. (B) Plot of the percent of monomer or dimer versus the logarithm of the total concentration of TnC at 40°C The solid lines represent monomer, the dotted lines represent dimer, the black lines represent TnC in the absence of TFE, and the gray lines represent TnC in the presence of TFE.

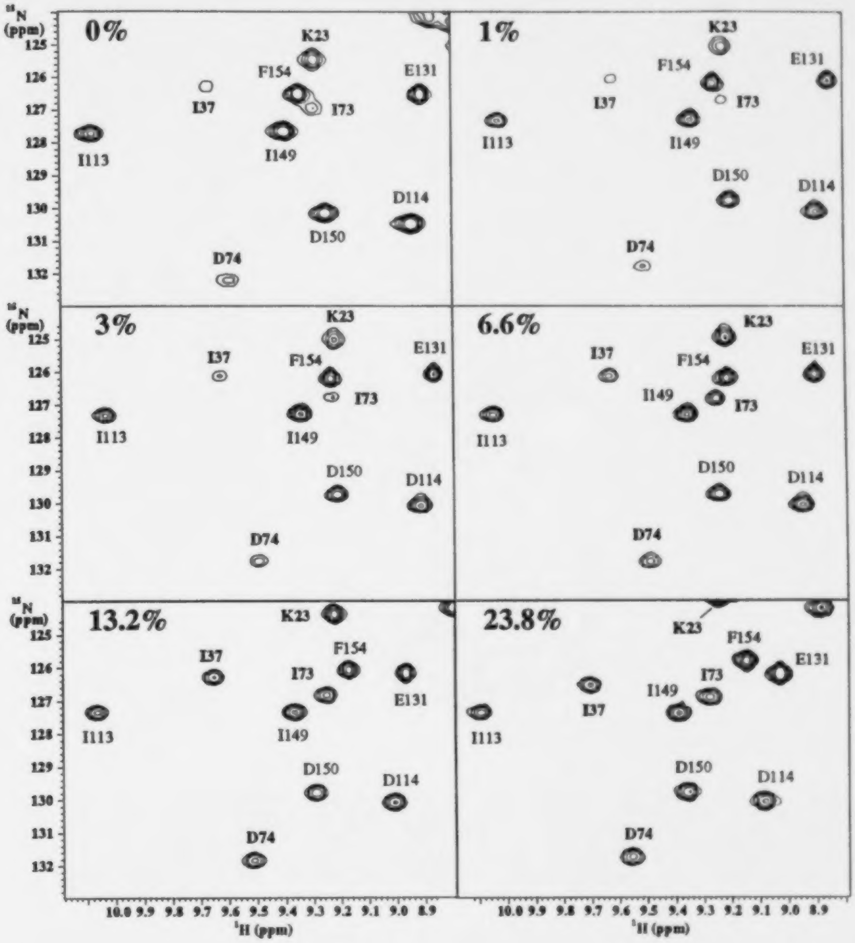


Figure V.3: A study of the effect of TFE on the intensity of crosspeaks in a series of 600 MHz 2D ^{1}H-^{15}N HSQC NMR spectra of uniformly ^{15}N-labeled TnC at a temperature of 40 °C. Shown is the portion of the HSQC spectrum corresponding to down-field shifted residues of TnC. Crosspeaks labeled in bold represent resonances from the N-domain, while those that are not correspond to resonances from the C-domain (for assignments see Chapter VI).

Measurement of ^{15}N or ^{13}C relaxation rates is useful for obtaining dynamic information since the relaxation of these nuclei is governed predominantly by the dipolar interaction with directly bound protons and to a much smaller extent by the chemical shift anisotropy mechanism (Allerhand et al., 1971), and thus these measurements provide a direct measure of mobility. Figure V.5 shows ^{15}N-T_2 relaxation studies on TnC in the presence of TFE. The average T_2 value for the N-domain is 83 ± 13 ms (residues 5 to 85) whereas the average T_2 value for the C-domain is 103 ± 18 ms (residues 95 to 158). The residues with the highest mobility are located at the C-terminus. The N-terminus

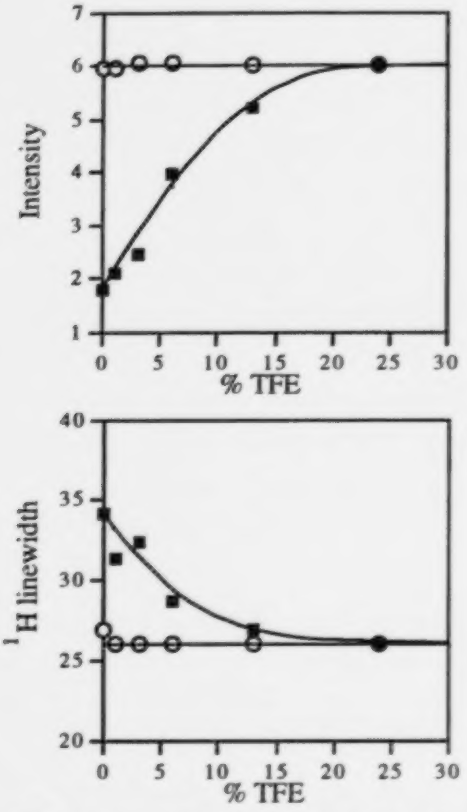


Figure V.4: (a) Plot illustrating the effect of TFE on the intensity of ^{1}H-^{15}N HSQC crosspeaks from the N-terminal and C-terminal domains. Shown are the crosspeaks from I37 (filled squares), representative of the N-domain, and I113 (open circles) which is representative of the C-domain. Residue I37 is in the N-domain calcium binding site I and is part of the β-sheet structure of the N-domain, and I113 has the same position in the C-domain in calcium binding site III. (b) Plot illustrating the effect of TFE on the ^{1}H linewidth of peaks from residues in the N-terminal and C-terminal domains. Shown are the crosspeaks from I37 (filled squares), representative of the N-domain, and I113 (open circles) which is representative of the C-domain.

exchanges too rapidly to observe, but most likely has mobility similar to the C-terminus. Residues K107 and K143 (residues in calcium binding sites III and IV of the C-domain) also appear quite flexible. Of particular importance are the residues in the linker region between the two domains (86 - 88) which are highly flexible, and allow the two domains to rotate freely about these residues. These data account for the differences in linewidths between the two domains, and explain the differential broadening of residues in the N-domain upon dimerization.

To determine the effects of TFE on the structure of TnC, two spectroscopic methods were employed; NMR and CD. One way with which to determine the secondary structure of a molecule is by the use of chemical shifts (Wishart et al., 1991 a,b; Wishart and Sykes, 1994a). In particular, the consensus of ^{13}Cα, CαH, and ^{13}CO chemical shift indices predicts to greater than 90% efficiency the secondary structure of a protein which is at least as good as having four inter-residue NOEs per residue in the protein (Wishart and Sykes, 1994a). Figure V.6 illustrates the chemical shift differences of ^{15}N, NH, CαH, ^{13}Cα, and ^{13}CO resonances between TnC in the presence and absence of TFE. In general, the chemical shift changes are small. As far as changes to secondary structure (in terms of the CαH, ^{13}Cα, and ^{13}CO resonances), V65, E76, E88, D89, and A90 incur the biggest changes. These changes, however, do not alter the secondary structure of these residues as determined by the chemical shift index method (Wishart and Sykes, 1994a). Of all of the chemical shifts, the greatest changes occur with residues valine 45, and methionine 48 (in helix B), leucine 49, and glycine 50 (in the B-C linker), glutamic acid 64, and valine 65 (in helix C), glutamic acid 76 (in helix D), glutamine 85, glutamic acid 88, aspartic acid 89, alanine 90, lysine 91 and glycine 92 (in the linker between the N and C domains). Smaller changes occur for residues threonine 4, alanine 8 and phenylalanine 13 (all in the N-helix), alanine 25 (in helix A), glutamic acid 57 (in helix C), phenylalanine 105 (in helix E), aspartic acid 106, and isoleucine 113 (in calcium binding site III), isoleucine 121 (in helix F), and valine 129 (in the linker between the F and G helices). These smaller changes, which generally affect only one chemical shift value, are most likely due to small changes in the hydrogen bonding character of the residue induced by TFE, or very small changes in the hydrophobic pocket due to the presence of TFE. The largest changes occur in the region at the end of helix B and the end of helix C, as well as in the linker between the two domains. The changes in helices B, and C are probably due to the break up of the dimer (since they are very, very broad resonances in the absence of TFE, especially residues around valine 65). The chemical shift change of residues in the linker region between the two domains of TnC could be due to some stabilization of the secondary structure in this region induced by TFE, but may also be due to the break up of the dimer.

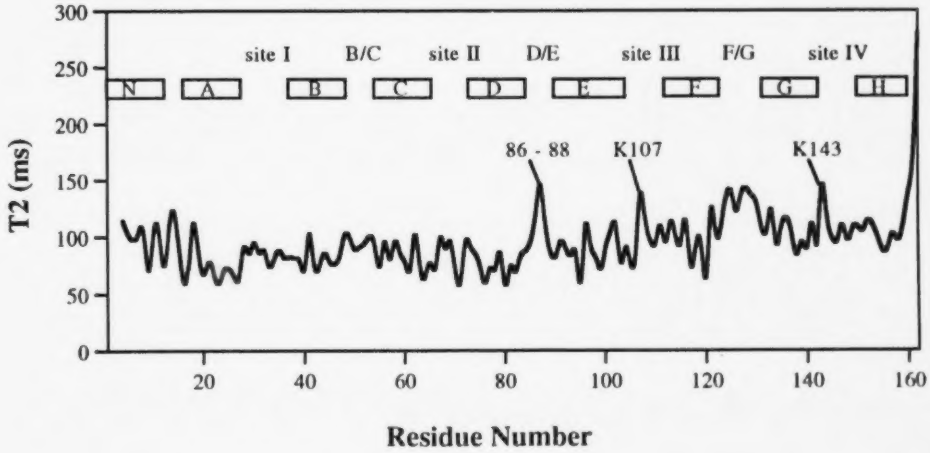


Figure V.5: Plot of the residue number versus the ^{15}N T_2 relaxation time for TnC in the presence of 15% TFE. Relaxation times not shown are for residues A1 to M3, D5, Q6, L49, P53, and H128. For overlapping resonances, it was assumed that the ^{15}N-T_2 relaxation time was the same for all. Indicated with the boxes are the helices of TnC. The calcium binding sites and linker regions are also indicated.

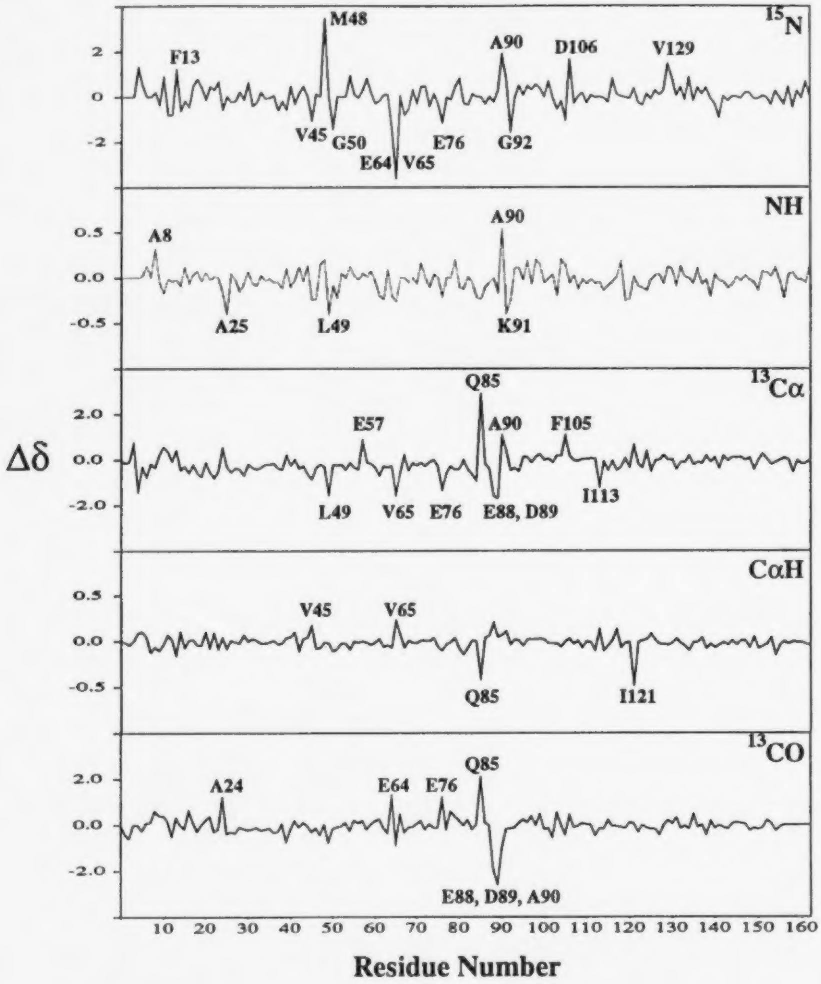


Figure V.6: The difference in the backbone NMR chemical shift data between TnC and TnC in the presence of 15% v/v TFE. The chemical shifts for TnC in TFE were assigned using a combination of heteronuclear multi-dimensional NMR experiments (see Chapter VI). Chemical shifts for the TnC dimer were obtained in the same manner as for TnC in TFE, except where discrepancies in the shift data were obtained (such as where the resonances were too broad or had too short of a T_2 relaxation time), shifts were obtained from the isolated N-domain (Gagné et al., 1995) or the isolated C-domain (Larry Calhoun, personal communication).

Figure V.7A represents the far UV-CD spectrum of TnC apo, calcium-saturated, and calcium saturated in the presence of TFE. The apo spectrum has a molar ellipticity of approximately -11 300 ± 500 (deg·cm^{2}·dmol^{-1}) at 220 nm. This value is similar to what has been obtained for recombinant TnC (Golosinska et al., 1991). When calcium is added to apo TnC, the negative molar ellipticity increases to approximately -16 400 ± 500 (deg·cm^{2}·dmol^{-1}) at 220 nm. This value is slightly lower by approximately 1000° than previously reported (Golosinska et al., 1991). The addition of TFE to calcium saturated TnC produces a further increase in negative molar ellipticity of approximately 1400° at 220 nm. This change could be due to a small increase in secondary structure, or due to the effects of tertiary or quaternary structural changes such as a slight rearrangement of helices (or break up of the dimer) (Manning, 1989).

Figure V.7B illustrates the near UV-CD spectrum of TnC apo, calcium-saturated, and calcium saturated in the presence of TFE. The spectra in the presence and absence of calcium or TFE show minima in ellipticity in the region of 250-270 nm associated with the major absorption bands of phenylalanine. The addition of calcium to TnC results in minor changes to the spectrum due to burying of the phenylalanine residues in the C-terminal domain hydrophobic pocket, as well as in the hydrophobic dimer interface. Addition of TFE to calcium saturated TnC produced minor changes most likely attributable to the break up of the dimer.

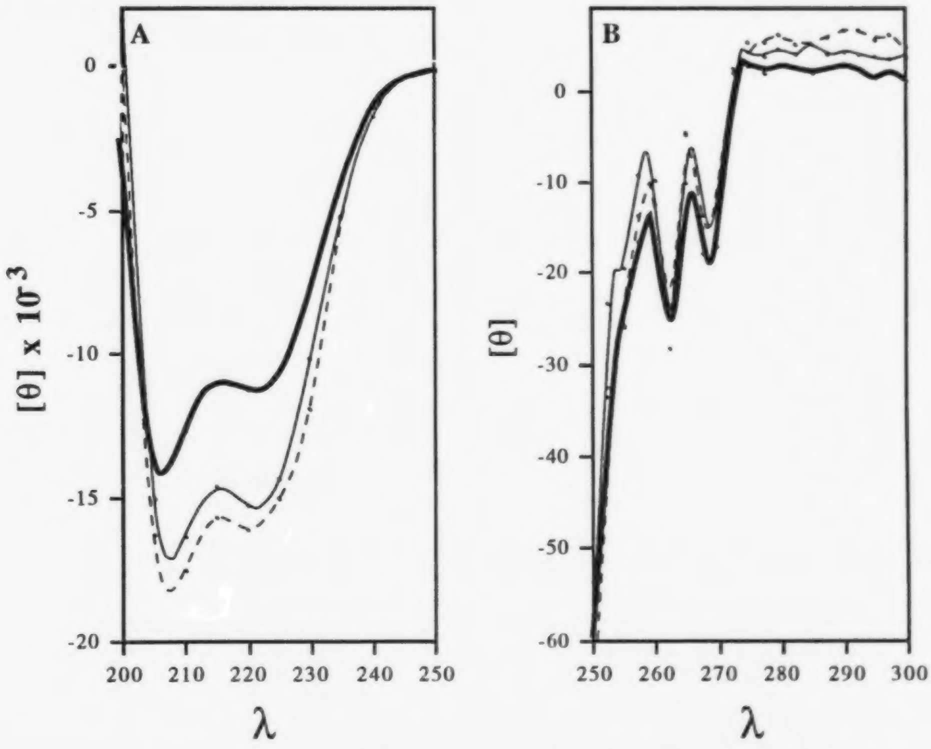


Figure V.7: (a) Far UV-CD spectra of apo TnC (dark solid line), calcium saturated TnC (light solid line), and calcium saturated TnC in the presence of 15% TFE (dashed line). Experimental conditions were as described in the Chapter III. (b) Near UV-CD spectra of apo TnC (dark solid line), calcium saturated TnC (light solid line), and calcium saturated TnC in the presence of 15% TFE (dashed line). Experimental conditions were as described in Chapter III.

Discussion:

^1H-^{15}N HSQC NMR spectra of calcium-saturated TnC at various temperatures gave the first clue that the mode of dimerization was via the N-domain as evidenced by differential line broadening of resonances from the N-domain at lower temperatures. To determine the structure of calcium-saturated TnC therefore required that the monomeric form be obtained either by raising the temperature or other intervention. The results shown here illustrate that at temperatures of 50°C and higher, some of the TnC structure was disrupted as some of the amide residues lost intensity at higher temperatures. Simply increasing the temperature was therefore not an option and it became necessary to find a solvent which would break apart the dimer without adversely affecting the tertiary structure of the monomer. We chose TFE for its known characteristic as a denaturant of quaternary structure (Lau et al., 1984 a,b) but keeping in mind that it could adversely affect the structure of the monomer.

A titration of TnC with TFE showed an increase in intensity with a concomitant decrease in linewidth of the N-domain NMR resonances while the C-domain NMR resonances were largely unaffected by the addition of TFE. Linewidth in NMR spectra are directly proportional to the rotational correlation time which, in turn, is directly proportional to the molecular weight. It may therefore be concluded that the molecular weight of the N-domain increased to a much greater extent than the molecular weight of the C-domain upon binding of calcium (and therefore dimerization of TnC as demonstrated by ultracentrifugation studies). This also suggests that there must be some sort of flexibility between the domains since the linewidths and intensities are so different for both domains. The ^{15}N-T_2 relaxation data supports the flexible linker between the two domains.

TnC is homologous in sequence and structure to another calcium binding protein, calmodulin. The solution structure of calmodulin illustrates the central region to be like a flexible tether between the two domains (Ikura et al., 1992). The ^{15}N NMR T_2 values for calmodulin were determined, and it was found that there was a difference in correlation times for both domains, and there was an increase in T_2 for six residues in the linker region between the two domains (Barbato et al., 1992). Our results, however, show an increase in the T_2 relaxation time for only three residues (M86 - K87 - E88), and these residues do not show the same increase in T_2 relaxation time as the residues in calmodulin. This implies that the linker is less flexible in TnC than calmodulin. This result is also supported by the work of Wang et al. (1993) who used fluorescence anisotropy decay measurements, and the work of Gulati et al. (1993) who studied mutants of the central helix.

To study the effects of TFE on secondary and tertiary structure, two types of spectroscopy (CD and NMR) were utilized. Far UV-CD spectroscopy revealed only a small change in negative ellipticity when TFE was added to calcium-saturated TnC. This change is most likely attributable to a breakup of the dimer interface since this region of the CD spectrum is sensitive not only to secondary structure, but to interactions amongst secondary structure elements (Manning, 1989). Near UV-CD also revealed very little change in the hydrophobic phenylalanine region when TFE was added to calcium-saturated TnC. The change observed could be attributed to an exposure of the hydrophobic pocket of the N-domain which contains the phenylalanine residues.

The chemical shifts of the backbone resonances are largely unaffected by the addition of TFE as was shown in Figure V.6. The ^{15}N and NH chemical shifts experience some of the greatest changes, however chemical shift changes of these nuclei are difficult to rationalize in terms of secondary structure due to helix dipole effects and amide chemical shift periodicities (Wishart and Sykes, 1994b). The chemical shift changes of the ^{13}Cα, CαH, and ^{13}CO resonances are, however, very good indicators of secondary structure. The shift downfield from random coil chemical shifts (positive deviations) of CαH resonances and the shift upfield from random coil chemical shifts (negative deviations) of ^{13}Cα and ^{13}CO signify the production of helical structure. The upfield shift of CαH, and the downfield shift of ^{13}Cα and ^{13}CO resonances signify the production of β-sheet structure. Although there are chemical shift deviations when TFE is added to TnC, these deviations do not affect the secondary structure, as the chemical shift index indicates the same secondary structure in the presence and absence of TFE (except for glutamine 85). A glance at Figure V.6 reveals that changes in chemical shift for all nuclei studied seem to be localized in the same regions of the molecule: Helix B, C and helix D/E which links the N and C domains together. The region of TnC linking the two domains together is largely exposed to solvent according to the crystal structure of half-saturated TnC, and thus when TFE is added, this region may have strengthened hydrogen bonds. ^{15}N-T$_2$ relaxation data indicate that residues 86 to 88 are mobile in the presence of TFE, and there is a difference in rotational correlation times of the two domains signifying that the effect of TFE in this region does not provide an uncharacteristically stable structure. The chemical shift changes in this region could therefore be due to the break up of the dimer as has been suggested for the changes in chemical shift of residues in helices B and C. The sidechain chemical shifts (data not shown) also experience minimal change with the addition of TFE indicating minimal, if any, changes to the tertiary structure of TnC.

A model for the calcium induced dimer based upon low angle x-ray scattering data has been presented (Blechner et al., 1992) which suggests that both the N and C-domains interact in a way similar to that seen in the crystal packing of TnC. The authors used Gly-92 in the interconnecting helix as a point of flexibility, allowing the N and C domains to rotate about this residue and tested random structures which would approximate the x-ray scattering data. The final model generated is not consistent with the results reported herein, as the C-terminal domain of each monomer is not flexible. Further, glycine 92 has not been shown to be flexible according to the T_2 data. The present study indicates that dimerization involves the N-domains from two monomers, and that the C-domains have minimal, if any, interaction with each other or the N-domain. Also, the interface for the dimer may involve helices B, C and possibly D/E, and residues M86 to E88 are flexible enough to cause the two domains to have different rotational correlation times.

The use of TFE, in this case, does not appear to alter the secondary or tertiary structure of TnC to any significant extent, due most likely to the fact that TnC has already formed its structure in the absence of TFE. This is a similar result to the one obtained for hen egg white lysozyme (Buck et al., 1993). Indeed, TFE has been shown to induce significant structure in peptides, or to alter the structure of peptides when compared to the aqueous solvent. We show here that the primary effect of TFE in small concentrations on proteins which have stable secondary and tertiary structures, is to break up the quaternary structure as was also shown for coiled coil peptides (Lau et al., 1984 a,b). Structure calculations of TnC in the presence of TFE are thus relevant as the change in secondary or tertiary structure of TnC is minimal at the low concentration of TFE used in this study. The use of any denaturing agent, however, should be used with caution. A complete study of the differences in secondary and tertiary structure should be done (in terms of CD and chemical shift information). The structure of TnC in TFE should provide an insight into the hydrophobic faces of TnC presented to other components of the thin filament as calcium is added, and hopefully give an indication as to how the domains relate to one another.

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Chapter VI:

Solution secondary structure of calcium-saturated troponin C monomer determined by multi-dimensional heteronuclear NMR spectroscopy.[1]

Troponin C (TnC) is the calcium-binding protein of the thin filament of muscle involved in the regulation of muscle contraction. Muscle contraction begins with the release of calcium from the sarcoplasmic reticulum. As the calcium concentration increases in the muscle cell, TnC binds calcium and undergoes a conformational change which is transmitted to other proteins in the thin filament leading ultimately to muscle contraction. This calcium-mediated regulation is of wide interest and many biochemical and biophysical studies have been undertaken to characterize the conformational change between calcium free and calcium bound TnC (for a review, see Grabarek et al., 1992). Chicken skeletal TnC has 162 amino acid residues (for a molecular weight of 18 257), and binds four metal ions in four separate metal ion binding sites numbered consecutively from the N-terminus of the protein. The structure of half-saturated avian skeletal TnC (with calcium binding sites III and IV filled) has been solved using x-ray crystallographic techniques (Satyshur et al., 1988, 1994; Herzberg and James, 1988) and reveals two domains (the C-terminal and the N-terminal domains), each with two EF-hand helix-loop-helix calcium binding sites. The C-terminal domain contains the high affinity calcium/magnesium binding sites which have a K_{Ca} in the range of 2×10^7 M^{-1}; these are referred to as the structural sites since they are always filled with calcium or magnesium in the muscle cell (Potter and Gergely, 1975). The N-terminal domain contains the lower affinity calcium specific binding sites which have a K_{Ca} in the range of 3×10^5 M^{-1}; these are referred to as the regulatory sites since the binding of calcium to these sites in skeletal muscle regulates muscle contraction

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[1] A version of this chapter has been accepted for publication. Slupsky, C.M., Reinach, F.C., Smillie, L.B., & Sykes, B.D. 1995. Protein Science 4.

(Potter and Gergely, 1975; Li et al., 1995). Knowledge of the structure of TnC in its half and fully calcium saturated forms is essential to understanding the molecular mechanism of muscle contraction.

A model for the calcium-induced conformational change for the N-domain of TnC has been proposed (Herzberg et al., 1986) based on the structure of the C-domain of TnC. This model suggests that when calcium binds to sites I and II, the major conformational transition is a movement of the B/C helix pair away from the A/D helix pair exposing a patch of hydrophobic residues providing a binding site for other muscle proteins. This model has strong support from several lines of evidence including NMR (Levine et al., 1977, 1978; Evans et al., 1980; Gagné et al., 1994) and site-directed mutagenesis (Fujimori et al., 1990; Grabarek et al., 1990; Gusev et al., 1991; Pearlstone et al., 1992a,b). There have been several studies of TnC which have focused on the function of the central helix (Babu et al., 1993; Gulati et al., 1990; Dobrowolski et al., 1991; Reinach and Karlsson, 1988; Xu and Hitchcock-DeGregori, 1988; Wang et al., 1993; Gulati et al., 1993). These studies have served to illustrate that the central helix of TnC is essential for TnC function, and that the helix is not as rigid as suggested by the crystal structure, although the long helix is extended in the model of the complex between TnC and TnI recently proposed from small-angle x-ray scattering (Olah and Trewhella, 1994). Calmodulin is a protein with very similar structural characteristics to TnC (Means and Dedman, 1980). Calmodulin has been shown to have a flexible linker between the two domains allowing the molecule to become more compact upon binding calcium (Barbato et al., 1992). TnC has also been reported to become more compact upon binding calcium most likely due to flexibility in the long central helix (Heidorn and Trewhella, 1988; Wang et al., 1993; Gulati et al., 1993). In order to elucidate the details of the structural change from the apo to the calcium saturated form of TnC, and to study the apparent flexibility of the central helix, we have initiated an NMR study of this protein.

Nuclear magnetic resonance (NMR) spectroscopy has developed into a technique capable of determining three dimensional structures of proteins in solution. The sequential resonance assignment of the backbone protons is the prerequisite for determining the three dimensional structure. Sequential assignment has most commonly been accomplished by means of homonuclear inter-residue through space correlations, coupled with homonuclear intra-residue through-bond correlations (Wüthrich, 1986). NMR of proteins has previously been limited to molecular weights less than 15 000 daltons due to the complexity of two-dimensional homonuclear spectra and the fact that linewidths increase rapidly as the size of the molecule increases. Developments in molecular biology have allowed not only large amounts of proteins to be expressed, but have allowed the incorporation of ^{15}N and ^{13}C isotope labels into proteins. The field of NMR has since exploded with a variety of three and four dimensional techniques which are extensions of two-dimensional techniques, allowing spectral editing and internuclear correlations based on the attached heteronuclei and can thus achieve better resolution than by two-dimensional methods. These methods may also be grouped into either NOESY-type (for example the ^{13}C-edited NOESY(Ikura et al., 1990b) or ^{15}N-edited NOESY (Marion et al., 1989a)), or COSY-type (for example the ^{15}N-edited TOCSY (Marion et al., 1989b), the CT-HNCA, CT-HNCO and CT-HN(CO)CA experiments (Grzesiek and Bax, 1992), the HCACO experiment (Powers et al., 1991), or the HCCH-COSY experiment (Bax et al., 1990a,b; Ikura et al., 1991b)). These new methods also represent new strategies for assignment of proteins based on the attached heteronucleus (Garrett et al., 1991; Meadows et al., 1994) and complement very well the "traditional" sequential assignment procedure. It is now feasible to solve the structure of proteins up to the size of 25 000 daltons (Clore and Gronenborn, 1991; Gronenborn and Clore, 1994; Wagner, 1993). Our goal has been to use these new techniques to solve the 3D solution structure of fully calcium saturated TnC.

TnC, at physiological pH, has previously been shown using ultracentrifugation techniques (Murray and Kay, 1972; Margossian and Stafford, 1982) to undergo a reversible calcium induced aggregation. At the concentration required for NMR observation, the calcium saturated state of TnC in water shows NMR resonance linewidths of the N-domain indicative of aggregation (Chapter V). Better linewidths can be achieved at high temperatures (Chapter V); however many resonances were lost due to amide exchange with the solvent. Varying pH and salt concentrations had minimal effect on reducing the aggregation problem (data not shown). The titration of calcium-saturated TnC with TFE revealed a set of resonances which were largely unaffected by the addition of TFE. These resonances corresponded to the C-terminal domain resonances. Another set of resonances, corresponding to the N-terminal domain resonances, experienced a decrease in ^{1}H linewidth and an increase in intensity upon addition of TFE (Chapter V). The interface for the dimer was shown to involve two N-terminal domains from two separate TnC monomers. Further, it was shown that residues located at the end of helix B and the end of helix C, which had significant changes in their chemical shifts, were possibly the residues involved in dimer interface (Chapter V). In the presence of 15% v/v TFE at 40°C, ultracentrifugation and NMR data illustrated that TnC behaves essentially like a monomeric unit (Chapter V).

The present NMR study deals with an over-expressed and isotopically labeled form of cloned chicken skeletal TnC in its monomeric form in the presence of 15% v/v TFE. Cysteine 101 of TnC was reacted with iodoacetamide to prevent oxidation and thus avoid aggregation. A detailed account the secondary structure of TnC derived from nuclear Overhauser enhancement (NOE) data, coupling constant information and chemical shift index data based upon the assignment of the backbone residues is presented. It will be shown that the secondary structure of TnC is minimally perturbed with use of TFE as a denaturant of quaternary structure, and that the TnC monomer secondary structure is essentially equivalent to the TnC dimer secondary structure. Finally, comparisons will be made to the x-ray crystal structure of half-saturated TnC.

Results:

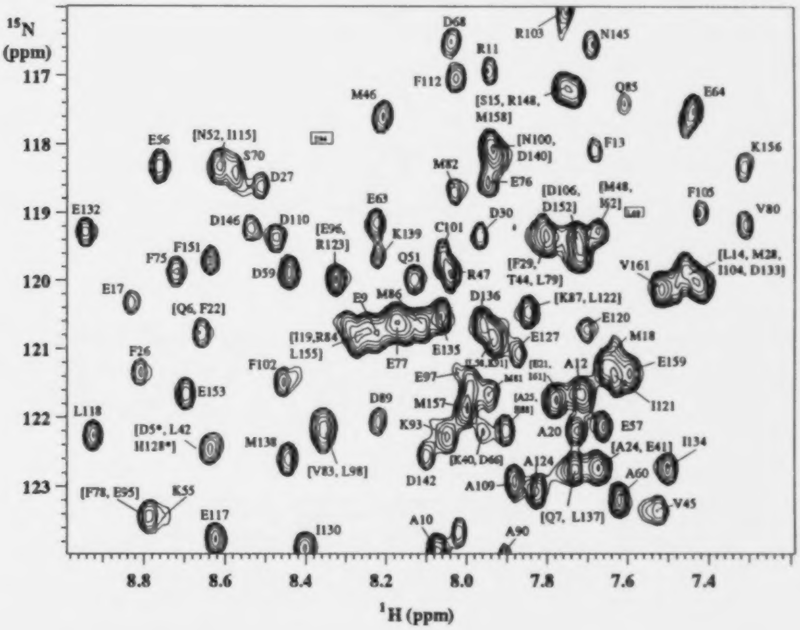

Figure VI.1: 2D ^1H - ^{15}N HSQC NMR spectrum of ^{15}N-labeled TnC showing the most crowded region of the spectrum. Those residues shown in square brackets are degenerate. Those residues indicated with an asterisk are tentative assignments. Boxes are placed in the spectrum where correlations were found using 3D techniques, but were not present in this spectrum.

Resonance Assignment strategy. Assignment of the ^1H, ^{13}C, and ^{15}N NMR resonances of the backbone nuclei of TnC made use of the four 3D triple resonance NMR experiments HNCA, HN(CO)CA, HNCO, and HCACO, in conjunction with the ^{15}N-edited NOESY and ^{15}N-edited TOCSY experiments. Figure VI.1 presents a portion of the ^1H-^{15}N-HSQC NMR spectrum of TnC in the presence of 15% TFE illustrating the most crowded region of the spectrum and the severe overlap in the ^{15}N and ^{1}H dimensions for this protein. There are 113 of the 158 observable amide to proton correlations in this region of the spectrum. Unobservable amide to proton correlations included A1, S2, and M3 which have fast amide proton exchange rates and one proline (P53) which does not have an amide hydrogen. Of these 113 correlations, 43 are overlapping. The residues which are overlapping are shown in square brackets in Figure VI.1. Fourteen correlations are composites of overlapping pairs of ^{15}N-^{1}HN crosspeaks, four correlations are three fold degenerate, and one correlation is four-fold degenerate. Most of the ^{1}H-^{15}N degeneracies could be resolved in at least one of either the HNCA, HN(CO)CA, or HNCO spectra. The ^{1}H-^{15}N assignments for D5 and H128 are tentative and based on a previous assignment of TnC in the absence of TFE, and these are shown by asterisks. These residues were not seen in any of the HNCA, HNCO or HN(CO)CA experiments possibly due to fast amide exchange. The resonances of L49 and S94 were unobservable in this 2D ^{15}N-^{1}H HSQC spectrum, and their positions are indicated in the figure. L49 and S94 were observed in the 3D HNCA, and the 3D HN(CO)CA spectra, whereas only L49 was observed in the 3D HNCO spectrum. Both were observed in the ^{15}N-edited NOESY spectrum and the ^{15}N-edited TOCSY spectrum.

Sequential assignment was achieved in several steps. Initially, possible amino acid spin systems were identified as arising from either the N-domain or the C-domain based on a calcium titration using 1D and 2D homonuclear NMR as a monitor (data not shown), and on previous partial assignments of TnC (Tsuda et al., 1988, 1990). These possible assignments were obtained by observing the differing exchange phenomena in the N- and C-terminal domains upon binding calcium ions. The C-terminal domain of TnC contains the high affinity calcium binding sites and the N-terminal domain of TnC contains the low affinity calcium binding sites (Potter and Gergely, 1975). During a calcium titration, where the calcium is increased from 0 to 2 mols of calcium per mol of protein, a signal corresponding to the unbound state gradually decreases in intensity with a concomitant increase in intensity of a signal at a different chemical shift value corresponding to the calcium bound state. Thus the C-domain exhibits slow chemical exchange. When the calcium concentration is increased from 2 to 4 mols of calcium per mol of protein, the signals gradually move across the spectrum, at each point the chemical shift value being an average of the bound and unbound states. Thus the N-domain exhibits fast chemical exchange. After identifying a few of these "starting places", the ^{1}HN and ^{15}N assignments were identified on an ^{15}N-^{1}H-HSQC NMR spectrum. Sequential connectivities along the backbone were then made using an approach similar to the one devised for sequential assignment of the homologous protein calmodulin (Ikura et al., 1990a) starting with the residues for which the ^{15}N and ^{1}H assignments were known. This approach involves combining the intra-residue $^{1}HN(i)$-$^{15}N(i)$-$^{13}C\alpha(i)$, $^{1}H\alpha(i)$-$^{13}C\alpha(i)$-$^{13}C'(i)$, and $^{1}HN(i)$-$^{15}N(i)$-$C\alpha H$ correlations with the sequential inter-residue $^{1}HN(i)$-$^{15}N(i)$-$^{13}C\alpha(i-1)$, $^{1}HN(i)$-$^{15}N(i)$-$^{13}C'(i-1)$, and $d_{NN}(i\pm1)$ correlations. Due to resonance overlap, many of the connectivities were not unique.

Figure VI.2 represents expansions of two-dimensional contour plots at selected ^{15}N frequencies of the 3D HNCA, HN(CO)CA, HNCO, ^{15}N-edited NOESY, and ^{15}N-edited TOCSY experiments, as well as selected $^{13}C'$ frequencies of the HCACO experiment illustrating the sequential assignment procedure using all six of these experiments. Details of the assignment can be found in the methods section, and assignment of all 162 residues may be found in Appendix B. In Figure VI.2, the sequential assignment for residues D150 to N145 are shown. For β-sheet regions the d_{NN} NOE is either very weak or unobservable (Wüthrich, 1986). Starting at residue D150 (which is in an antiparallel β-sheet structure in calcium binding site IV), the HNCA spectrum shows an intense crosspeak for the intra-residue correlation and a weak crosspeak for the inter-residue correlation. At the same ^{15}N (130.0 ppm) and ^{1}HN (9.29 ppm) frequencies, the $^{13}C\alpha(i-1)$ correlation (to I149) may be found in the HN(CO)CA spectrum, and the $^{13}C'(i-1)$ assignment (to I149) may be found in the HNCO spectrum. The $^{13}C\alpha(i-1)$, and $^{13}C'(i-1)$ frequency information obtained from D150 for I149 may be used to search the HCACO experiment to find the $^{1}H\alpha$ frequency of I149. Since there are no NOESY $d_{NN}(i\pm1)$ correlations, ^{15}N planes must be searched in the ^{15}N-edited TOCSY and HNCA experiments, and all ^{1}HN frequencies that correlate the $^{1}H\alpha$ and $^{13}C\alpha$ chemical shifts of I149 must be found. Fortunately this procedure yielded only one possibility, and therefore the sequential assignment procedure could be continued from I149 in the same way to obtain the ^{15}N, ^{1}HN, $^{13}C\alpha$, $^{13}C'$, and $^{1}H\alpha$ chemical shift values for R148. A $d_{NN}(i-1)$ is observable in the ^{15}N-edited NOESY spectrum of R148 making the assignment from R148 to G147 easier since ^{15}N planes at a particular ^{1}HN frequency need only be sampled. Very weak crosspeaks are found for G147 Hα atoms (and all glycine Hα atoms) in the HCACO experiment due to the fact that they are only weakly excited because they are so far upfield in the Cα region. The TOCSY transfer of magnetization to G147 α-protons was also very poor possibly due to the fact that G147 is very far downfield (10.38 ppm) in the ^{1}HN dimension. Assignment of G147 (even without the presence of the $^{1}H\alpha$ chemical shifts in the ^{15}N-edited TOCSY) could be continued because a $d_{NN(i,i-1)}$ from R148 to G147 was found and only one $d_{NN(i,i+1)}$ could be found in all the ^{15}N planes of the ^{15}N-

edited NOESY to link G147 to R148. At this ^{15}N and ^{1}HN frequency, the $^{13}C\alpha$ shift for G147 found on the HNCA corresponded to the $^{13}C\alpha$ shift suggested for G147 by R148. The assignment from G147 to D146, and D146 to N145 using $d_{NN}(i\text{-}1)$, $^{13}C\alpha(i\text{-}1)$, $^{13}C'(i\text{-}1)$, and $^{1}H\alpha(i\text{-}1)$ information obtained from the experiments was straightforward. Using all six experiments, the complete assignment of the backbone chemical shifts for TnC were obtained, and are shown in Table VI.1.

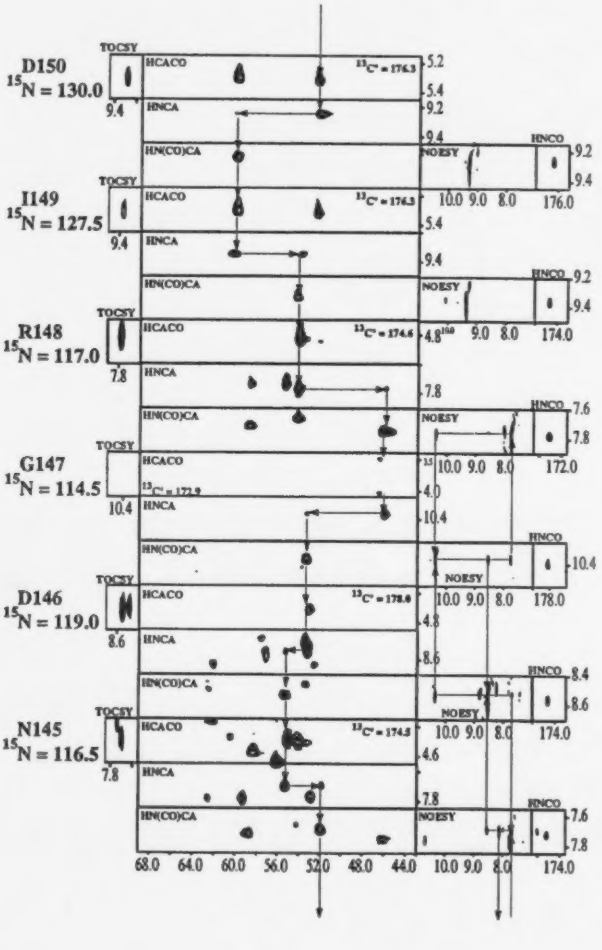

Figure VI.2: ^{1}H - $^{13}C\alpha$ planes extracted from HCACO, HNCA, HN(CO)CA, and HNCO experiments, and ^{1}H-^{1}H planes extracted from ^{15}N-edited TOCSY, and ^{15}N-edited NOESY spectra at selected ^{15}N (HNCA, HN(CO)CA, HNCO, ^{15}N-edited TOCSY, and ^{15}N-edited NOESY), and $^{13}C'$ (HCACO) frequencies showing the sequential assignment for a set of six residues (D150 to N145). Shown are the $^{13}C\alpha$-^{1}HN correlations of the HNCA and HN(CO)CA, the $^{13}C\alpha$-$^{1}H\alpha$ correlations for the HCACO, the $^{13}C'$-^{1}HN correlations for the HNCO, the ^{1}HN-^{1}HN correlations for the ^{15}N-edited NOESY and the ^{1}HN-$^{1}H\alpha$ correlations for the ^{15}N-edited TOCSY.

Table VI.1: Backbone Chemical Shifts of Resonances of skeletal troponin C.

	chemical shift (ppm)						chemical shift (ppm)				
residue	^{15}N	NH	^{13}Cα	CαH	^{13}CO	residue	^{15}N	NH	^{13}Cα	CαH	^{13}CO
A1			52.1	4.12	174.6	D59	119.9	8.46	57.5	4.34	179.0
S2			58.3	4.57	174.5	A60	123.2	7.65	55.1	4.19	180.1
M3			55.3			I61	121.7	7.80	65.5	3.68	178.0
T4	114.8	8.03	62.4	4.35	175.0	I62	119.2	7.68	65.1	3.49	177.8
D5	122.6*	8.66*	57.6	4.34	179.0	E63	119.1	8.24	59.5	4.03	178.6
Q6	120.8	8.72	59.6	4.02	179.0	E64	117.6	7.46	59.1	4.11	178.2
Q7	122.8	7.80	58.2	4.04	177.7	V65	112.0	7.49	62.2	4.36	176.2
A8	124.2	8.19	55.2	4.20	180.5	D66	122.3	7.98	54.1	4.62	176.9
E9	120.8	8.16	58.6	4.14	178.4	E67	129.5	8.55	58.9	4.30	177.2
A10	123.8	8.07	54.8	4.14	178.8	D68	116.6	8.05	52.7	4.73	177.8
R11	117.1	7.96	59.0	3.78	177.8	G69	110.2	7.74	47.2	3.92, 3.81	175.4
A12	121.1	7.72	53.7	4.20	179.1	S70	118.5	8.59	60.2	4.23	176.2
F13	118.2	7.70	58.7	4.54	175.4	G71	117.6	10.79	45.7	4.12, 3.44	172.6
L14	120.0	7.44	54.0	4.40	176.0	T72	108.7	7.70	58.3	4.90	173.7
S15	117.3	7.70	56.6	4.65	175.2	I73	126.9	9.27	60.6	5.07	176.2
E16	123.2	9.09	60.6	3.96	179.0	D74	131.8	9.48	53.2	5.27	176.0
E17	120.4	8.85	60.1	4.05	179.0	F75	119.9	8.74	61.8	3.63	176.5
M18	120.9	7.66	58.6	4.07	178.4	E76	118.4	7.94	60.0	3.89	179.2
I19	120.8	8.27	67.3	3.76		E77	120.6	8.20	58.5	4.20	179.0
A20	122.2	7.76	55.2	4.10	181.1	F78	123.5	8.80	61.5	4.05	176.7
E21	121.8	7.77	59.6	4.20	179.9	L79	119.3	7.82	58.4	3.42	178.7
F22	120.8	8.68	59.5	4.94	178.8	V80	119.1	7.33	67.4	3.35	178.0
K23	124.6	9.22	58.6	4.02	177.4	M81	121.7	7.95	59.2	3.81	178.2
A24	122.8	7.70	54.4	4.15	179.6	M82	118.9	8.05	56.7	4.11	179.2
A25	122.2	7.94	55.2	4.07	178.3	V83	122.3	8.41	67.3	3.63	178.3
F26	121.3	8.82	62.6	3.17	176.9	R84	120.9	8.25	60.4	3.84	179.0
D27	118.6	8.55	57.2	4.20	178.2	Q85	117.5	7.60	54.4	4.58	175.8
M28	120.0	7.43	58.3	4.08	178.0	M86	120.4	8.00	58.0	4.21	177.5
F29	119.4	7.81	58.8	4.23	177.9	K87	120.4	7.88	57.8	4.11	177.1
D30	119.2	7.98	52.3	4.51	176.9	E88	122.3	7.94	58.6	4.14	178.4
A31	130.8	7.71	55.2	4.08	179.3	D89	122.1	8.24	56.1	4.57	177.5
D32	114.4	8.25	53.0	4.59	177.9	A90	124.0	7.93	53.8	4.25	179.0
G33	111.6	8.00	47.0	3.86	175.9	K91	120.9	7.92	58.1	4.21	178.0
G34	109.6	8.16	46.5	4.08, 3.98	176.1	G92	110.0	8.23	46.0	4.01	175.2
G35	113.8	10.69	45.3	4.47, 3.65	173.3	K93	122.2	8.08	57.3	4.41	177.6
D36	116.2	7.78	52.8	5.16	173.4	S94	117.9	8.36	59.7	4.48	175.7
I37	126.4	9.64	60.2	4.96	176.0	E95	123.7	8.80	60.4	4.08	178.4
S38	124.1	8.77	56.0	4.87	175.9	E96	120.1	8.32	59.6	4.11	179.1
T39	115.8	9.09	66.6	3.77	177.7	E97	121.6	8.04	59.6	4.18	179.9
K40	122.3	7.93	59.6	4.07	179.9	L98	122.2	8.37	58.4	4.34	179.5
E41	122.7	7.71	58.6	4.04	179.5	A99	124.5	8.61	55.5	4.12	179.9
L42	122.2	8.64	58.1	4.09	178.8	N100	118.2	7.96	56.5	4.46	177.4
G43	106.6	8.62	48.2	3.88, 3.64	175.4	C101	119.5	8.12	59.0	4.09	175.5
T44	119.5	7.80	67.5	3.90	176.3	F102	121.4	8.47	62.5	3.32	176.3
V45	123.0	7.53	67.0	3.50	177.8	R103	116.1	7.78	59.2	4.00	178.4
M46	117.6	8.23	59.6	4.00	178.9	I104	120.0	7.45	64.1	3.65	177.7
R47	120.0	8.07	59.1	4.69	181.4	F105	119.1	7.47	58.2	4.42	177.0
M48	119.2	7.72	59.0	4.22	177.7	D106	119.5	7.74	52.4	4.48	177.1
L49	119.0	7.58	55.1	4.37	177.7	K107	127.5	7.62	59.2	4.00	178.1
G50	108.3	7.84	45.8	4.26, 3.77	174.8	N108	115.0	8.03	51.8	4.72	174.4
Q51	120.0	8.14	54.2	4.51	174.4	A109	122.9	7.89	53.2	4.08	176.6
N52	118.0	8.60	51.2	5.16	172.2	D110	119.5	8.48	53.3	4.70	177.5
P53			62.8	4.76	177.6	G111	113.7	10.42	45.2	3.99, 3.38	172.4
T54	113.5	8.48	60.4	4.52	175.5	F112	117.0	8.03	56.0	5.44	175.0
K55	123.2	8.76	60.4	3.88	178.1	I113	127.4	10.07	59.8	4.96	176.1
E56	118.4	8.77	60.8	4.09	180.0	D114	130.2	9.00	52.3	4.98	176.5
E57	122.2	7.70	59.5	4.00	179.3	I115	118.4	8.64	64.8	3.97	177.3
L58	120.9	7.94	58.1	4.04	178.8	E116	125.1	7.93	59.5	4.12	180.3

Table VI.1: Backbone Chemical Shifts of Resonances skeletal troponin C - Continued

	chemical shift (ppm)						chemical shift (ppm)			
residue	^{15}N	NH	^{13}Cα	CαH	^{13}CO	residue	^{15}N	NH	^{13}Cα	CαH	^{13}CO
E117	123.8	8.66	59.2	4.07	179.8	D140	118.2	7.96	56.8	4.48	177.6
L118	122.3	8.94	57.9	4.01	178.9	S141	114.2	7.75	60.4	4.49	174.3
G119	106.0	8.13	47.9	3.99, 3.65	175.5	D142	122.7	8.09	53.1	4.62	176.8
E120	120.9	7.64	59.7	4.10	179.3	K143	126.6	8.02	57.0	4.24	177.8
I121	121.3	7.61	64.6	3.49	178.8	N144	115.4	8.13	51.7	4.85	175.0
L122	120.4	7.87	57.9	4.02	180.2	N145	116.8	7.71	55.1	4.49	174.5
R123	120.0	8.34	59.4	4.15	179.1	D146	119.2	8.55	53.1	4.71	178.0
A124	123.1	7.84	54.3	4.28	179.2	G147	114.3	10.38	46.0	4.02, 3.47	172.9
T125	108.6	7.63	63.0	4.32	175.9	R148	117.1	7.77	54.1	4.83	174.6
G126	110.4	7.80	45.6	4.15, 3.88	174.2	I149	127.4	9.37	60.0	5.30	176.3
E127	121.1	7.91	56.1	4.31	176.2	D150	129.8	9.29	52.5	5.32	176.3
H128	122.5*	8.66*	56.2	4.64	174.5	F151	119.9	8.66	62.0	3.72	176.5
V129	124.4	7.83	61.8	4.26	175.4	D152	119.4	7.75	58.1	4.19	179.9
I130	123.9	8.41	60.4	4.50	177.3	E153	121.8	8.71	59.0	4.00	180.1
E131	126.1	8.93	61.1	3.85	178.6	F154	126.1	9.17	61.5	4.27	176.7
E132	119.4	8.96	60.0	4.07	178.0	L155	120.8	8.28	58.4	3.57	180.1
D133	119.9	7.47	56.9	4.53	178.8	K156	118.2	7.31	58.5	4.03	177.4
I134	122.8	7.53	65.0	3.58	177.6	M157	121.8	8.03	58.7	4.08	179.2
E135	120.5	8.07	60.2	3.85	179.0	M158	117.2	7.78	55.3	4.35	178.2
D136	120.5	7.97	57.7	4.40	178.2	E159	121.4	7.52	58.4	4.08	177.9
L137	122.7	7.75	58.0	4.22	180.5	G160	108.4	7.98	45.7	4.06, 3.91	174.4
M138	122.7	8.46	58.4	4.26	177.6	V161	120.2	7.56	62.8	4.14	175.4
K139	119.4	8.24	59.6	4.10	179.3	Q162	129.3	7.73	57.7	4.18	180.7

^{1}H Chemical shifts are referenced to internal DSS at 0.0 ppm, ^{13}C Chemical shifts are referenced to external DSS at 0.0 ppm, and ^{15}N Chemical shifts are referenced to external $^{15}NH_4Cl$ at 24.93 ppm. Chemical shifts are for E. coli expressed chicken skeletal troponin C (1.4 mM) (modified at cysteine 101 with a carboxamidomethyl group) in 150 mM KCl, 16 mM $CaCl_2$, 15% v/v TFE at pH 7.0 and 40°C. Residues marked with a "*" are tentative assignments.

Secondary Structure Determination. Secondary structure elements in proteins have distinct NOE connectivity patterns as described by Wüthrich (1986). Helices may be characterized by the existence of $d_{\alpha N}(i,i+3)$, $d_{\beta N}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$, and strong $d_{NN}(i,i+1)$ connectivities, whereas β-sheets are characterized by strong $d_{\alpha N}(i,i+1)$ and the absence of $d_{NN}(i,i\pm1)$ connectivities. A new method for characterizing helices is the ratio of the $d_{N\alpha}/d_{\alpha N}$ (Gagné et al., 1994) (where $d_{\alpha N}$ refers to the NOE involving the Hα of residue i-1 to the HN of residue i, and $d_{N\alpha}$ refers to the NOE between HN of residue i and Hα of residue i). α-helices are characterized by a $d_{N\alpha}/d_{\alpha N}$ ratio greater than one, whereas β-sheets are characterized by a $d_{N\alpha}/d_{\alpha N}$ ratio less than one. In a simulation where spin-diffusion is taken into account and compared to the results using a two-spin approximation (data not shown), it was observed that all $d_{N\alpha}/d_{\alpha N}$ ratios tended to unity as the amount of spin-diffusion increased regardless of the type of secondary structure. It follows, therefore, that the measured $d_{N\alpha}/d_{\alpha N}$ ratio underestimates the amount of α-helix or β-sheet present. In addition to the ratio of the $d_{N\alpha}/d_{\alpha N}$, there is a strong correlation between the deviations of $^{13}C\alpha$, $^{13}C'$ and $^{1}H\alpha$ chemical shifts from their random-coil values with the secondary structure of the protein (Wishart et al., 1991 a,b). For α-helices, positive deviations from random coil values occur for the $^{13}C\alpha$ and $^{13}C'$ shifts and negative deviations occur for the $H\alpha$ shifts. For β-sheets, negative deviations from random coil values occur for the $^{13}C\alpha$ and $^{13}C'$ shifts and positive deviations occur for the $^{1}H\alpha$ shifts. α-helices may also be characterized by small HN-Hα J couplings due to the smaller dihedral angle between the HN and Hα protons whereas β-sheets have large HN-Hα J couplings due to the larger dihedral angle between the HN and Hα protons. Because of significant overlap in the ^{15}N-edited NOESY spectrum, four of these data (the $d_{\alpha N}(i,i+3)$ NOE's, the $d_{N\alpha}/d_{\alpha N}$ ratio, the chemical shift index, and the J-coupling data) taken together were used to define the secondary structure of TnC as illustrated in Figure VI.3. Also shown are the slowly exchanging amide hydrogens which indicate buried or hydrogen bonded amide hydrogens. Of the unambiguously assigned residues, the slowly exchanging amide resonances are F26, A20, D30, I37, L42, V45, M46, E63, I73, D74, E77, M82, F105, G111, F112, I113, D114, L118, I149, D150, E153, and F154. The slowest exchanging amide resonances are F26, F105, F112, L118, and F154. This data is similar to that obtained for calmodulin (Ikura et al., 1991a) where it was found that the calcium-binding loops of the C-terminal domain exchange slower than the calcium-binding loops of the N-terminal domain suggesting that the calcium-binding loops of the C-terminal domain are more stable in their conformation than the calcium binding loops of the N-terminal domain.

The data in Figure VI.3 identify the location of nine helices, four calcium binding loops, and four short β-strands. The helices span the regions (N) Asp 5 - Phe 13, (A) Glu 16 - Phe 29, (B) Thr 39 - Met 48, (C) Lys 55 - Glu 64, (D) Phe 75 - Arg 84, (E) Asp 89 - Phe 105, (F) Glu 116 - Ala 124, (G) Glu 131 - Ser 141, and (H) Phe 151 - Gly 160. The beginnings and endings of the helices are marked by changes in one or more of the chemical shift index, the $d_{N\alpha}/d_{\alpha N}$ ratio, and the J-coupling. The only ambiguity occurred in the central region between the two domains where it appears that the secondary structure is mainly helical, however T_2 data (Chapter V) have shown that three residues (M86 - E88) are flexible. Q85 appears to have a CSI and $^{3}J_{HNH\alpha}$ indicative of β-sheet type secondary structure. The helices were thus broken up at R84 and restarted at D89. The crystal structure of half-saturated TnC reveals similar starts and stops of helices (Herzberg and James, 1988; Satyshur et al., 1988, 1994) except for the ends of some helices and in the linker region between the two domains where the helix is contiguous in the crystal structure (from F75 to F105). One residue of particular interest, is the residue Glu 41. This residue was found to have 'irregular' non-helical phi/psi angles (ϕ = -96°; ψ = -7°) in the crystal structure and was shown to form a "kink" in helix B of the crystal structure where the N-domains are free of calcium (Herzberg and James, 1988; Satyshur et al., 1988, 1994). The calcium-saturated TnC structure presented here has no indication of phi/psi angles for residue Glu 41 other than helix, suggesting that this residue has shifted its phi/psi angles to better fit into a helix in the calcium-saturated form. This result was also verified for the calcium-saturated isolated N-domain, NTnC (Gagné et al., 1994).

The four β-sheet regions span residues 36 to 38, 72 to 74, 112 to 114, and 148 to 150. The β-sheets are in an antiparallel conformation (36 to 38 is antiparallel to 72 to 74, and 112 to 114 is antiparallel to 148 to 150) as observed by the presence of $d_{\alpha\alpha}$, d_{NN}, and $d_{\beta N}$ NOEs across the β-sheet ($d_{\alpha\alpha}$ for D36 to D74, S38 to T72 (these $^1H\alpha$ resonances overlap), F112 to D150, and D114 to R148; d_{NN} for I37 to I73 and I113 to I149; and $d_{\beta N}$ for I37 to I73 and I113 to I149) as was also observed for the symmetric homodimers in solution (Shaw et al., 1990; Kay et al., 1991). The calcium binding loops are well characterized by x-ray crystallographic studies (Herzberg and James, 1988; Satyshur et al., 1988, 1994), and consist of twelve residues flanked by α-helices in the typical EF-hand configuration. The calcium binding loops are comprised of the residues (I) Asp 30 - Glu 41, (II) Asp 66 - Glu 77, (III) Asp 106 - Glu 117, and (IV) Asp 142 - Glu 153. The first four residues in the C-terminal calcium binding loops (D106 - A109 and D142 - N145) are in a type I turn conformation as evidenced by strong NOE's between K107 NH (or K143 NH) to N108 NH (or N144 NH), N108 NH (or N144 NH) to A109 NH (or N145 NH), and K107 Hα (or K143 Hα) to A109 NH (or N145 NH). The equivalent residues in the N-terminal domain (D30 - G33 and D66 - G69), however, do not exhibit the $d_{\alpha N(i,i+2)}$ NOE's of the $H\alpha_{(i+1)}$ to the $NH_{(i+3)}$ residues in the loop.

There are four connecting loops in calcium saturated TnC. These comprise residues Leu 14 - Ser 15 (between the N-helix and helix-A), residues Gln 51 - Thr 54 (between helix-B and helix-C), Gln 85 - Glu 88 (between helix D and helix E), and residues Thr 125 - Ile 130 (between helix-F and helix-G). T_2 data (Chapter V), suggest that the only flexible linker region is that between helices D and E, as well as F and G. The linker in the N-domain (between helices B and C) does not appear flexible on the time scale of the T_2 experiment. Similar results were obtained for calmodulin which has similar linker regions (Barbato et al., 1992).

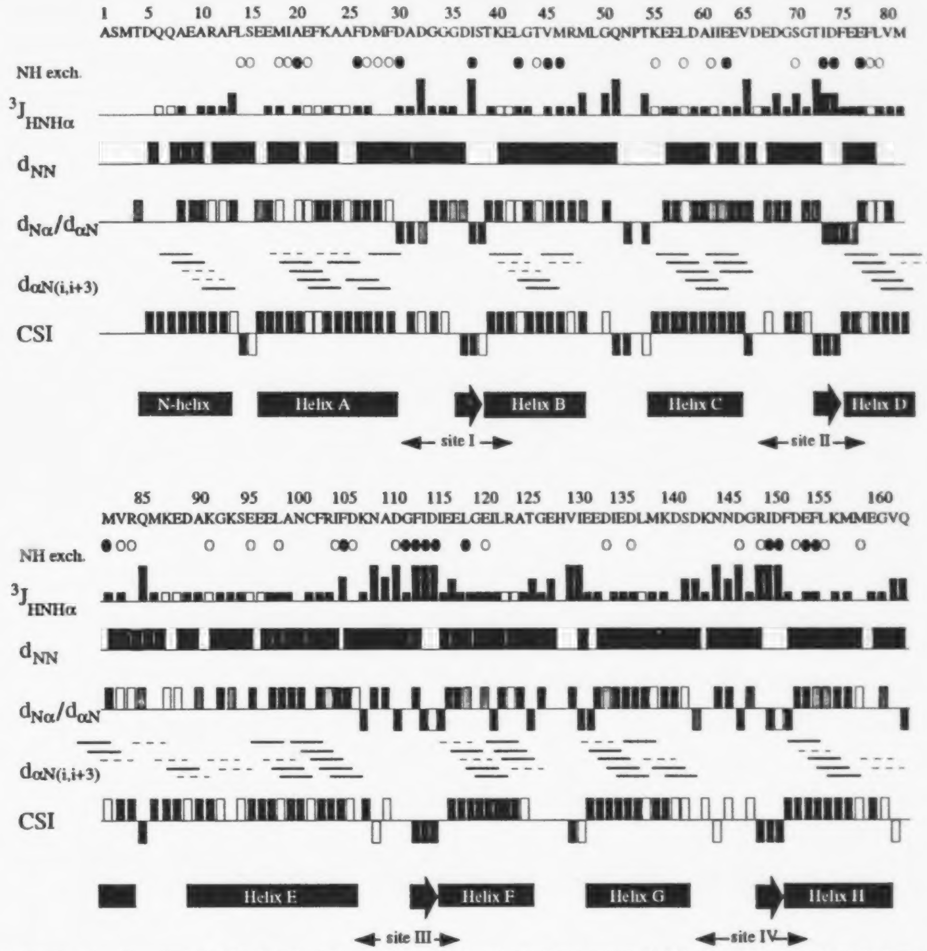

Figure 3: Summary of the TnC data on sequential and medium range NOEs involving amide protons, the ratio of the $d_{N\alpha}/d_{\alpha N}$ intensities, the $^3J_{HNH\alpha}$ couplings, and the chemical shift index. The $d_{N\alpha}/d_{\alpha N}$ ratio represents the ratio between the $d_{N\alpha(i,\ i)}$ and the $d_{\alpha N(i-1,\ i)}$ NOE intensities. The boxes pointing up represent a ratio >1, and the boxes pointed down represent a ratio <1. Unfilled boxes represent degeneracies in either the $d_{N\alpha(i,\ i)}$ or the $d_{\alpha N(i-1,\ i)}$ with other residues or with itself. Gray boxes represent ratios for which either the $d_{N\alpha(i,\ i)}$ or the $d_{\alpha N(i-1,\ i)}$ are missing, and it is assumed that the intensities are very small. No box means either the ratio is approximately equal to 1, or that there was not enough information to determine the ratio (ie. both the $d_{N\alpha}$ and $d_{\alpha N}$ are absent). The $^3J_{HNH\alpha}$ values are shown only if they are unambiguous (filled boxes) or ambiguous (unfilled boxes) when it is clear that no splitting can be observed. For the CSI, the filled boxes pointing up indicate that all three chemical shift values ($^{13}C\alpha$, $^{13}C'$, and $^1H\alpha$) represent helix, the filled boxes pointing down indicate that all three chemical shift values ($^{13}C\alpha$, $^{13}C'$, and $^1H\alpha$) represent β-sheet, and no box represents random coil chemical shifts. Unfilled boxes represent CSI values for which 2/3 represent that particular secondary structure. For the $d_{\alpha N(i,i+3)}$, the dotted lines represent an ambiguity. The secondary structure elements are marked according to the criteria shown where boxes represent α-helix and arrows represent β-sheet. Unambiguous slowly exchanging amide resonances are shown with filled circles, whereas ambiguous slowly exchanging amide resonances are shown with unfilled circles. Residues and their numbering are indicated above.

Discussion

The data presented provide the first structural data of fully calcium saturated TnC. In determining the secondary structure of TnC, a combination of the $d_{\alpha N}(i,i+3)$, the chemical shift index, the J-coupling data, and the $d_{N\alpha}/d_{\alpha N}$ ratio were used. The distance corresponding to the $d_{N\alpha}$ NOE is virtually the same for both the α-helix and the β-sheet (between 2.7 Å, and 3.05 Å) where 96% of the distances corresponding to the $d_{N\alpha}$ NOE are in this range and 96% of the distances corresponding to the $d_{\alpha N}$ NOE are out of this range (Gagné, S.M., personal communication). The distance corresponding the $d_{\alpha N}$ NOE varies depending on the type of secondary structure. The $d_{\alpha N}$ NOE in β-sheets is more intense than in α-helices since the α-proton of one residue is very close to the amide proton of the next residue (a distance of between 1.8 and 2.5 Å in β-sheets). α-helices, however, have a much smaller intensity for the $d_{\alpha N}$ (the distance in α-helices between the NH(i) and CαH(i-1) is between 3.3 and 3.6 Å). The $d_{N\alpha}/d_{\alpha N}$ ratio should thus provide us with a method for determining the secondary structure independent of the ^{15}N amide exchange rate or ^{15}N T_2 of that particular residue. The consensus of the chemical shift index ($^{13}C\alpha$, $^{13}C'$, and $^{1}H\alpha$ chemical shift deviations from random coil values) can predict to greater than 90% accuracy the secondary structure of a protein (Wishart et al., 1994). The CSI, the $d_{N\alpha}/d_{\alpha N}$ ratio, the $d_{\alpha N}(i,i+3)$ NOE's and the J-coupling data therefore provides a very accurate determination of the secondary structure of TnC which is summarized in Figure VI.3.

Comparison of the secondary structure of TnC with that of the crystal structure of TnC reveals striking similarities. The nine helices start and stop at approximately the same residue (Herzberg and James, 1988; Satyshur et al., 1988; Satyshur et al., 1994). The major difference in secondary structure occurs with the residue Glu 41 which is located in helix B. In the crystal structure, and in the secondary and tertiary structure of the apo N-domain (Gagné et al., 1994, 1995), helix B appears to have a break or kink at residue E41 (irregular phi-psi angles). The B helix is thus started at residue 39, and continues to residue 40, breaks, then continues again at residue 42 as shown in Table VI.2. In the structure of calcium saturated TnC and calcium-saturated NTnC (Gagné et al., 1994), E41 does not have irregular phi or psi angles, but is in a helical conformation (as supported by the d_{NN} NOEs, the $^{3}J_{HNH\alpha}$ coupling constants, as well as the CSI) (Table VI.2). The other differences are at the end of helix B which occurs one residue sooner in the NMR derived structure versus the crystal structure, and the four linker residues between the two domains found in the NMR structure. The observation of subtle changes in the N-domain

Table VI.2: Comparison of α-helices.

	Residue Range			
	TnC - $4Ca^{2+}$	TnC - $2Ca^{2+}$	CaM - $4Ca^{2+}$	NTnC - $2Ca^{2+}$
N-Helix	5 - 13	3 - 13	n/a	5 - 13
Helix A	16 - 29	16 - 28	6 - 19	16 - 29
Helix B	39 - 48	39 - 40, 42-48	29 - 38	39 - 48
Helix C	55 - 64	55 - 64	45 - 54	55 - 64
Helix D	75 - 84	75 - 105	65 - 77	75 - 87
Helix E	89 - 105	75 - 105	82 - 93	n/a
Helix F	115 - 124	115 - 125	102 - 111	n/a
Helix G	131 - 141	131 - 141	118 - 127	n/a
Helix H	151 - 160	151 - 159	138 - 146	n/a

TnC - $4Ca^{2+}$ are helices found in the present study of calcium saturated TnC. The helix range was based on the $^3J_{HNH\alpha}$, CSI, $d_{\alpha N}(i,i+3)$ NOE, and the $d_{N\alpha}/d_{\alpha N}$ NOE ratio. TnC - $2Ca^{2+}$ are from Herzberg and James (1988). The helix limits were based on expected $^3J_{HNH\alpha}$ and NOE's from the crystal structure. CaM - $4Ca^{2+}$ are helices as described in Ikura et al. (1991). NTnC - $2Ca^{2+}$ are helices as described in Gagné et al. (1994).

on going from half to fully saturated TnC was first realized by Levine et al. (1977). They deduced from NMR studies at 270 MHz, that calcium binding to the low affinity sites causes changes in the environment of largely hydrophobic residues. The results were later verified by Levine et al. (1978), Evans et al., (1980), and Gagné et al. (1994).

The secondary structure of calmodulin has been determined (Ikura et al., 1991a) using NMR and it was found that the secondary structure is very similar to that obtained for TnC. By aligning the calcium binding loops, very similar secondary structural elements and helix lengths were found. The numbering for calmodulin is different from the numbering for TnC by ten residues in the N-domain (due to the lack of an N-terminal helix in calmodulin), and thirteen residues in the C-domain (due to a difference of three residues in the linker between the two domains). In the calcium binding loops, there exists a short antiparallel β-sheet of three residues in both calmodulin and TnC. Table VI.2 illustrates the differences in the helices. For the most part, the helices in both calcium-saturated TnC and calmodulin are the same. The only difference occurs in the linker region where calmodulin has six flexible residues versus three flexible residues for TnC (although Q85 has phi dihedral angles indicative of β-sheet structure, the ^{15}N T_2 data does not support flexibility for this residue). A comparison of residues in homologous positions in calmodulin and TnC reveals a 46% identity. Directly comparing the chemical shifts of these identical residues reveals that 81% of the backbone $^{13}C\alpha$ and $^{13}C'$ shifts are within 1 ppm and ^1H shifts are within 0.1 ppm. Including the backbone ^{15}N chemical shift (with an error of 1 ppm), the chemical shift similarity drops to 63%. For sidechain ^1H, ^{15}N and ^{13}C chemical shifts, there is 75% identity between TnC and the identical residue in calmodulin (assuming a 1 ppm error for ^{13}C and ^{15}N chemical shifts and 0.1 ppm error for ^{1}H chemical shifts).

The secondary structure of the N-terminal domain of TnC is also very homologous to that obtained for the isolated N-domain (Gagné et al., 1994). The helix lengths are virtually identical, and the calcium binding loops are as well (Table VI.2). In terms of the similarity of assignment, 85% of the backbone chemical shifts and 87% of the sidechain chemical shifts of N-TnC are identical with TnC (with the same errors as for the Calmodulin comparison). The SCIII homodimer (Shaw et al., 1992) has 46% homology for the backbone chemical shifts and 50% for the sidechain chemical shifts, and the TH2 homodimer (Kay et al., 1991) has 44% for the backbone chemical shifts and 58% for the sidechain chemical shifts.

In this paper, we have described the details of the backbone ^{1}H, ^{13}C and ^{15}N resonance assignments of TnC on the basis of the analysis of the 3-dimensional triple resonance experiments. These assignments were shown to be highly homologous to assignments made for calcium saturated N-TnC (Gagné et al., 1994), as well as to the structurally homologous protein calmodulin. Certainly this high homology suggests very similar secondary structure in terms of backbone chemical shifts, but also suggests very similar tertiary structure as illustrated by the high homology in the sidechain chemical shifts. Secondary structure analysis of TnC confirms the supposition that the secondary structure of TnC is similar to these other structures. The elucidation of the three-dimensional structure of TnC should confirm the similarity in tertiary structure of TnC to these other structures. Finally, it should be pointed out that the use of 15% v/v TFE to break up aggregation of TnC has not adversely affected the secondary or tertiary structure of TnC, thus reinforcing the use of TFE as a general perturbant of quaternary structure for stable proteins.

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Chapter VII:

The three-dimensional structure of calcium-saturated troponin C.

Troponin C (TnC), an 18 kDa protein, is the calcium-sensitive molecular trigger for muscle contraction. TnC contains two spatially independent calcium binding domains and is present in both fast twitch (skeletal) and cardiac muscles. TnC is a member of a trimer of proteins called the troponin complex (TnI and TnT being the other two components) which is anchored onto the actin filament and tropomyosin via the TnT subunit. Release of calcium by the sarcoplasmic reticulum results in the binding of calcium to TnC and the release of the inhibition of the actin-myosin interaction.

The crystal structure of skeletal TnC with its structural (C-terminal) calcium binding sites filled and the regulatory (N-terminal) calcium binding sites apo (2-Ca^{2+}-TnC) has been solved (Herzberg and James, 1988; Satyshur et al., 1988, 1994). The C-terminal domain contains the high affinity calcium/magnesium binding sites which have a K_{Ca} in the range of $2x10^7$ M^{-1} (Potter and Gergely, 1975). These sites have typically been referred to as the structural sites since they are believed to always be filled with calcium or magnesium in the muscle cell. The N-terminal domain contains the low affinity calcium-specific binding sites which have a K_{Ca} in the range of $3x10^5$ M^{-1} (Potter and Gergely, 1975; Li et al., 1995). These sites are referred to as the regulatory sites since binding of calcium to these sites regulates muscle contraction (see Chapter I).

There have been many studies trying to deduce exactly what the conformational change of TnC is upon binding calcium. It has been suggested, using circular dichroism as a monitor for calcium binding, that the α-helical content of the N-terminal domain of TnC increases upon addition of calcium (Murray and Kay, 1972; Pearlstone et al., 1992a,b; Li et al., 1994; Chandra et al., 1994). In contrast, ^1H NMR studies on proteolytic fragments of TnC have revealed that calcium binding to the low affinity sites results in subtle alterations of the tertiary fold (Evans et al., 1980; Hincke et al., 1981; MacLachlan et al., 1990; Krudy et al., 1992). Hydrophobic interaction chromatography has suggested that the binding of calcium to the TnC low affinity sites results in the opening of a hydrophobic patch which should be more similar to the two domains of calmodulin, than to its own C-terminal domain (Vogel et al., 1983).

A model for the calcium-induced conformational change of the N-terminal domain has been proposed (Herzberg et al., 1986) based on the structure of the C-terminal domain of TnC. The model suggests that when calcium binds to calcium binding sites I and II, the major conformational transition is a movement of the B/C helix pair away from the N/A/D helices to expose a patch of hydrophobic residues that will provide a binding site for other muscle proteins. Several studies, using site-directed mutagenesis (Fujimori et al., 1990; Grabarek et al., 1990; Gusev et al., 1991; Pearlstone et al., 1992a,b), cysteine reactivity in cardiac TnC (Ingraham and Hodges, 1988; Fuchs et al., 1989; Putkey et al., 1993), or NMR studies (Lin et al., 1994; Gagné et al., 1994) have shown that, in general, the model is a good approximation of the actual structure of calcium-saturated TnC in solution. It was shown, however, that while the model may generally be correct, there are problems with the structural details (Pearlstone et al., 1991b; Gagné et al., 1994).

Recently, the 3D solution structures of apo NTnC and calcium saturated NTnC have been solved (Gagné et al., 1995). These structures confirm previous findings and illustrate that not only does the secondary structure of the N-terminal domain remain constant upon calcium binding, but that the calcium-saturated N-terminal domain is more open than suggested by the model structure (Gagné et al., 1995). As shown in Chapter V, the N-terminal domain is involved in the dimerization of TnC at saturating calcium concentrations, and consequently calcium-saturated NTnC should be a dimer in solution. It is therefore important to study TnC in its monomeric form to be sure that the dimer interface does not introduce artifacts into the three-dimensional structure.

There have been a number of questions raised as to the stability of the central helical region of TnC presented in the half saturated crystal structures. Solution x-ray scattering studies have suggested that TnC is more compact at physiological pH and saturating calcium concentrations (Wang et al., 1987; Hubbard et al., 1988; Heidorn and Trewhella, 1988). This result has been verified using monoclonal antibodies (Strang and Potter, 1990), fluorescence resonance energy transfer (Wang et al., 1993), and site-directed mutagenesis (Reinach and Karlsson, 1988; Gulati et al., 1990, 1993; Dobrowolski et al., 1991; Babu et al., 1993; Ding et al., 1994). Calmodulin, a protein with very similar structural characteristics to TnC, has also been shown to have a flexible central helix (Barbato et al., 1992). Chapter V indicated, with the use of ^{15}N-T_2 measurements, that the central helix of TnC is flexible, but not as flexible as calmodulin. In order to understand and elucidate the details of the structural change from apo to calcium-saturated TnC in its monomeric form and to study the flexibility of the central helix, we have initiated an NMR study of this protein.

This chapter presents the sidechain ^{1}H, ^{13}C, and ^{15}N assignments, and describes the three-dimensional structure of TnC with all four calcium binding sites filled which has been determined using multidimensional heteronuclear nuclear magnetic resonance (NMR) spectroscopy, in the presence of 15% v:v TFE to alter dimerization in favor of monomer. As will be shown, comparison of this calcium saturated structure with the x-ray crystallographic structure (Herzberg and James, 1988; Satyshur et al., 1988, 1994) of half-saturated skeletal TnC will reveal two major differences. The first difference is an opening of the hydrophobic pocket in the N-domain upon binding calcium, and the second difference involves residues of the inter-domain linker where residues 85 to 94 have conformational heterogeneity resulting in an imprecise spatial relationship between the two domains. The use of 15% v:v TFE as a denaturant of quaternary structure (see Chapter V) has no effect on the folding of TnC as evidenced by similar structural characteristics between the C-terminal domain of this structure and that of the x-ray crystallographic structure as well as between the N-terminal domain of this structure and that of the NTnC dimer (Gagné et al., 1994). The elucidation of the structure of the calcium-saturated form of TnC is the first step to understanding the link in the signal between TnC and the formation of myosin-actin cross-bridges. These results are also important for understanding the general mechanism of calcium activation in this class of calcium-binding proteins.

Results and Discussion:

Sidechain Assignment: The ^{1}H, ^{13}C, and ^{15}N NMR chemical shifts of skeletal troponin C were assigned using double and triple resonance three-dimensional NMR experiments on samples of uniformly ^{15}N and ^{15}N/^{13}C-labeled protein (see Chapter III for experimental details). Backbone assignment of calcium-saturated TnC was accomplished as described in Chapter VI. Sidechain assignment involved the combined analysis of 3D HCCH-COSY, ^{13}C-edited NOESY, ^{15}N-edited NOESY and 2D ^{1}H-^{1}H DQF-COSY and NOESY experiments. The first step in sidechain assignment involved identifying $^{13}C_\alpha$, and $^1H_\alpha$ resonance chemical shifts on an HCCH-COSY experiment and correlating these with the $^1H_\beta$ resonance. Each plane on the HCCH-COSY spectrum was then examined to find the symmetrically related $^{13}C_\beta/^1H_\beta$ peak correlating the $^1H_\beta$ resonance to the $^1H_\alpha$ resonance. This procedure was continued along the sidechain finding symmetrically related peaks correlating ^{1}H resonances and their attached ^{13}C frequencies with ^{1}H resonances on adjacent carbon atoms. Figure VII.1 illustrates an HCCH-COSY spectrum where the assignment of T72 and I130 is shown. Assignment of isoleucine 130 was initiated by finding the ^{13}C plane correlating the $^{13}C_\beta/^1H_\beta$ resonance with the $^1H_\alpha$ and γ-methyl and methylene protons. The HCCH-COSY spectrum was subsequently surveyed to find the symmetric peaks from the $^{13}C_\gamma/^1H_3C_\gamma$ to the $^1H_\beta$ (to obtain the ^{13}C assignment of the γ-methyl carbon) and the $^{13}C_\gamma/^1H_2C_\gamma$ to the $^1H_\beta$ and to the $^1H_3C_\delta$ protons. For assignment of the $^{13}C_\delta$, the symmetric peak correlating $^{13}C_\delta/^1H_3C_\delta$ to the γ-methylene protons were found. This figure illustrates the ease at which the sidechain assignment was obtained. Also shown in Figure VII.1 is the assignment of T72 of the N-terminal domain. Assignment of all residues was verified by finding similar peaks on the ^{13}C-edited NOESY spectrum. Assignment of the aromatic rings of TnC (11 phenylalanine residues) was done by a combination of 2D DQF-COSY and NOESY experiments in D_2O, where connectivities through the ring were found on the DQF-COSY spectrum and assignment to a particular residue was made from observation of an NOE from the δ-ring protons to the $^1H_\beta$ protons. Assignment of sidechain amide resonances (from asparagine and glutamine residues) was accomplished using the ^{15}N-edited NOESY experiment, where NOE's from the amide to H_β or H_γ protons were found. The complete sidechain assignment (^{1}H, ^{13}C, and ^{15}N) of calcium-saturated TnC is given in Table VII.1.

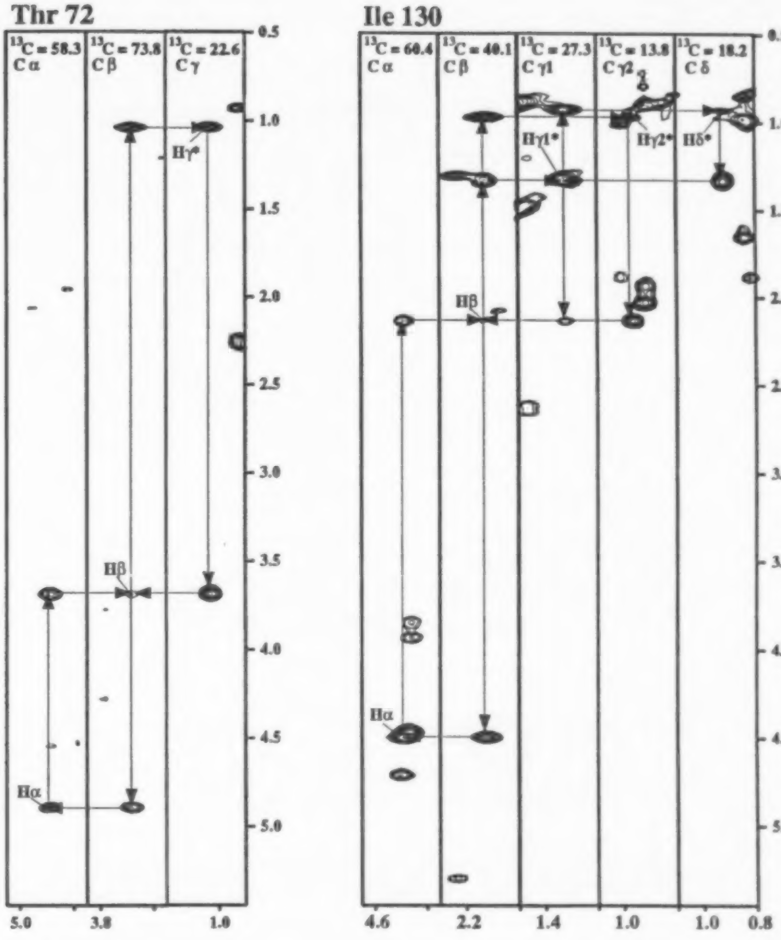


Figure VII.1. Sidechain assignment of threonine 72 located in the N-terminal domain of TnC, and isoleucine 130 located in the C-terminal domain of TnC. Shown are strips taken from different ^{13}C planes (^{13}C-frequency is indicated at the top of the strip) of the HCCH-COSY spectrum illustrating how sidechain assignment was accomplished using this experiment.

Table VII.1: Side-Chain Chemical Shifts of Resonances of skeletal troponin C.

residue	^{13}Cβ	Hβ	^{13}Cγ	Hγ	^{13}Cδ	Hδ	^{13}Cε	Hε	others
A1	20.0	1.56							
S2	64.4	3.90							
M3							16.9	2.07	
T4	69.9	4.29	21.7	1.20					
D5	40.5	2.73, 2.62							
Q6	31.2	2.25, 2.06		2.44, 2.34					
Q7									
A8	17.8	1.55							
E9	30.4	2.07	36.1	2.31, 2.21					
A10	17.7	1.42							
R11		1.84	28.3	1.92, 1.71	44.0	3.21			
A12	19.0	1.35							
F13	40.9	3.19, 3.14				7.28		7.37	
L14	44.4	1.97, 1.57	27.8	1.90	26.04, 23.87	0.88, 0.92			
S15	65.9	4.39, 4.01							
E16	29.6	2.07	37.5	2.36					
E17	29.1	2.07, 1.95	37.8	2.50, 2.30					
M18	32.6	2.14, 1.93	32.7	2.48, 2.35			16.7	1.98	
I19	37.8	2.04	29.1, 17.7	1.64, 0.98	13.8	0.85			Hγ2* 1.15
A20	18.2	1.52							
E21									
F22	38.3	3.56, 3.49				7.18		7.34	
K23	32.2	1.91		1.20	27.4	1.46, 0.38	42.3	2.65, 2.62	
A24	19.0	1.46							
A25	17.8	1.72							
F26	40.1	2.77				6.74		7.12	Hζ 7.29
D27		2.64, 2.61							
M28		2.15, 2.09	32.4	2.63, 2.40			16.9	2.09	
F29	40.1	2.90, 2.70				7.14		7.28	
D30	38.9	2.46, 1.46							
A31	19.9	1.54							
D32	40.2	3.11, 2.72							
G33									
G34									
G35									
D36	41.7	3.04, 2.29							
I37	40.1	1.88	27.4, 18.1	1.21	15.2	0.45			Hγ2* 1.01
S38	66.8	4.48, 4.03							
T39	68.6	4.17	23.4	1.36					
K40	33.0	1.93, 1.78	25.2	1.47, 1.41	29.5	1.70	42.3	3.00	
E41									
L42	42.7	1.94, 1.50	27.0	1.61	26.2, 23.1	0.85, 0.85			
G43									
T44	69.2	4.35	21.6	1.27					
V45	31.7	1.95	23.0, 20.8	0.66, 0.36					
M46	30.4	2.07, 1.81	33.5	2.71, 2.49			17.3	1.93	
R47	30.3	1.99, 1.94	28.9	1.93, 1.81	43.7	3.23			
M48	32.2	2.38, 2.27	32.2	2.78, 2.66			17.3	2.20	
L49	42.2	1.90, 1.78			21.7	0.86, 0.86			
G50									
Q51	30.5	2.15, 1.66	33.1	2.23	182.6			7.18, 6.65	Nε 112.52
N52	39.6	2.82, 2.53	176.7			7.48, 6.69			Nδ 113.18
P53	32.6	2.18, 1.96		2.02	50.5	3.67, 3.27			
T54	72.0	4.71	22.3	1.36					
K55	32.6	1.96, 1.80	25.2	1.50	29.5	1.72	41.8	2.99	
E56		2.81, 1.95	37.4	2.50, 2.30					

Table VII.1: Side-chain Chemical Shifts of Resonances of skeletal troponin C - Continued

residue	^{13}Cβ	Hβ	^{13}Cγ	Hγ	^{13}Cδ	Hδ	^{13}Cε	Hε	others
E57									
L58		2.12	27.8	1.80	27.0, 22.6	0.84, 0.79			
D59	40.5	2.73, 2.62							
A60	18.3	1.53							
I61	38.4	1.96	30.5, 17.3	1.84, 1.15	13.0	0.89			Hγ2* 0.82
I62	37.1	2.04	29.5, 17.0	1.68, 1.17	12.1	0.73			Hγ2* 0.72
E63	29.6	2.16, 2.08							
E64	33.5	2.15, 2.09		2.64, 2.58					
V65	33.5	2.26	22.6, 20.4	0.92, 0.97					
D66	41.4	2.69							
E67		2.24, 2.06		2.32, 2.24					
D68	40.9	3.13, 2.76							
G69									
S70	65.0	4.22, 4.03							
G71									
T72	73.8	3.68	22.6	1.04					
I73	40.6	2.04	27.1, 18.5	1.59, 0.90	14.1	0.86			Hγ2* 1.29
D74	41.8	3.38, 2.81							
F75	38.8	2.48, 2.08				6.70		7.11	Hζ 7.39
E76		1.96, 1.89							
E77									
F78	40.4	3.44, 3.23				7.04		7.28	
L79		1.64, 1.13	26.4	1.16	25.6, 24.4	0.57, 0.67			
V80	31.7	2.23	23.4, 21.6	1.05, 0.88					
M81		2.25, 2.01							
M82	32.1	1.51, 1.22							
V83	31.7	2.08	23.9, 21.7	1.08, 0.78					
R84	30.0	1.88	28.3	1.74, 1.62	43.6	3.18			
Q85	28.7	2.17	34.3	2.44, 2.36	182.0			7.24, 6.50	Nε 112.0
M86	32.2	2.38, 2.27	32.6	2.77, 2.64			17.3	2.20	
K87			26.1	1.27, 1.22		2.48, 2.17			
E88		2.07, 1.87							
D89	41.0	2.76, 2.69							
A90		1.36							
K91	30.0	2.26, 2.15		2.44, 2.35					
G92									
K93	33.5	1.90, 1.84		1.49	28.9	1.66, 1.56		2.97	
S94	64.3	4.16, 4.02							
E95		2.18							
E96									
E97		2.26							
L98		2.23, 2.06	26.6	1.83	25.6	0.83, 0.83			
A99	18.6	1.40							
N100	38.4	3.01, 2.86	177.7			7.67, 6.94			Nδ 114.3
C101	35.6	3.38, 3.18							
F102	40.5	3.14, 2.78				6.44		6.93	Hζ 7.20
R103	30.5	1.92	28.9	1.93, 1.81	43.7	3.23			
I104	37.9	1.80	29.1, 17.0	1.62, 1.10	13.0	0.76			Hγ2* 0.66
F105	38.8	3.02, 2.55				7.41		7.32	Hζ 7.26
D106	39.1	2.47, 1.47							
K107	33.4	1.92	25.2	1.66, 1.55	29.2	1.80	42.2	3.07	
N108	37.1	3.30, 2.84	179.0			7.79, 6.72			Nδ 113.6
A109	17.3	1.37							
D110	41.4	3.24, 2.47							
G111									

Table VII.1: Side-chain Chemical Shifts of Resonances of skeletal troponin C - Continued

residue	^{13}Cβ	Hβ	^{13}Cγ	Hγ	^{13}Cδ	Hδ	^{13}Cε	Hε	others
F112	44.4	2.77, 2.73				6.93		7.40	Hζ 7.34
I113	39.2	1.92	27.4, 18.2	1.05, 0.51	14.2	0.35			Hγ2* 0.92
D114	42.3	3.38, 2.58							
I115	38.8	1.92	30.1, 17.7	1.49, 1.40	14.7	0.92			Hγ2* 1.00
E116	30.5	2.17, 1.93		2.32, 2.07					
E117	30.1	2.48		2.85					
L118	41.8	1.81, 1.43	27.0	1.80	26.0, 23.0	0.86, 0.82			
G119									
E120	29.9	2.25, 2.10		2.41					
I121	37.5	1.88	27.8, 16.8	1.31, 0.42	13.2	0.50			Hγ2* 0.46
L122	44.4	1.81, 1.47	27.8	1.82	25.7, 23.9	0.83, 0.83			
R123	30.6	1.91	29.2	1.84, 1.60		3.24, 3.18			
A124	18.7	1.62							
T125	70.7	4.40	21.3	1.36					
G126									
E127	30.9	1.96, 1.75	36.1	2.22, 2.16					
H128	30.0	3.15, 3.07							
V129	34.0	1.96	22.1, 20.8	0.86, 0.80					
I130	40.1	2.13	27.3, 18.2	1.33	13.8	0.93			Hγ2* 0.97
E131	29.7	2.06, 2.04		2.36, 2.35					
E132	29.7	1.99	37.3	2.39, 2.30					
D133	41.0	2.94, 2.67							
I134	37.9	2.03	29.1, 18.2	1.65	13.8	0.86			Hγ2* 0.92
E135	29.6	2.09, 2.05		2.33, 2.23					
D136	41.0	2.83, 2.68							
L137	41.9	1.86, 1.67	27.4	1.57	26.4, 23.6	0.89, 0.91			
M138	33.8	2.21, 2.18	31.8	2.63, 2.50			17.4	2.07	
K139	32.6	1.96	25.3	1.66, 1.52	29.1	1.66	43.1	3.03	
D140	38.3	2.97, 2.82							
S141	65.0	3.93, 3.84							
D142	40.1	2.99, 2.53							
K143			25.6	1.53, 1.67		1.98, 1.74			
N144	37.5	3.31, 2.81	178.7			7.85, 6.68			Nδ 113.8
N145	37.9	3.07, 2.69	178.2			7.37, 6.67			Nδ 112.9
D146	41.3	2.97, 2.40							
G147									
R148	34.5	1.63, 1.50	26.0	1.27, 1.22	43.2	2.47, 2.16			
I149	40.0	2.24	28.3, 18.5	1.04	14.2	0.92			Hγ2* 1.30
D150	42.7	3.32, 2.93							
F151	39.1	2.56, 2.27				6.61		7.17	Hζ 7.28
D152	41.4	2.75, 2.63							
E153									
F154	40.2	3.43, 3.28				7.11		7.38	Hζ 7.42
L155	41.8	1.72, 1.28	26.5	1.20	26.1, 24.4	0.64, 0.68			
K156	30.5	1.88		1.58, 1.56	28.2	1.84, 1.55	44.0	3.10, 3.03	
M157	32.4	2.16	32.4	2.59, 2.40			17.7	1.98	
M158	32.3	1.85, 1.74					17.2	2.05	
E159	31.4	2.05		2.42, 2.24					
G160									
V161	32.8	2.16	21.7, 21.2	0.93, 0.95					
Q162	30.9	2.11, 1.93	34.7	2.31	181.6			7.52, 6.88	Nε 112.5

^{1}H chemical shifts are referenced to internal DSS at 0.0 ppm, ^{13}C chemical shifts are referenced to external DSS at 0.0 ppm, and ^{15}N chemical shifts are referenced to $^{15}NH_4Cl$ at 24.93 ppm. Chemical shifts are for E. coli expressed chicken skeletal troponin C (1.4 mM) (modified at cysteine 101 with a carboxamidomethyl group) in 150 mM KCl, 16 mM $CaCl_2$, 15% v/v TFE at pH 7.0 and 40°C.

Quality of TnC Structure: The three-dimensional structure of calcium-saturated skeletal TnC was determined using a simulated annealing protocol from a total of 2,282 NMR-derived restraints (Nilges et al., 1988a,b,c) (see Chapter III). The structures all satisfy the distance restraints with no violations greater than 0.35 Å, and no dihedral violations greater than 5°. Details of how the distance and angular information were obtained may be found in Chapter III. The distance, ϕ and ψ restraints used for the structure calculations may be found in Appendix C, and the XPLOR input files used to generate the structures as well as analyze the structures are given in Appendix D. A superposition of 23 NMR-derived structures is shown in Figure VII.2, and the structural statistics are given in Table VII.2. Residue-based rms deviations for these structures are shown in Figure VII.3, and angular order parameters are shown in Figure VII.4. As shown in Table VII.2, the NMR structures exhibit good covalent geometries (as indicated by the low rms deviations from idealized values for bonds, angles and impropers, as well as low values for the dihedral, NOE, vanderWaals, and total energies calculated with the use of a vanderWaals repulsion term of 0.75). In addition, the large negative Lennard-Jones vanderWaals energy indicates that there are no bad nonbonded contacts.

Shown in Figure VII.2 A and B is a superposition of the 23 structures of the N-terminal and C-terminal domains onto the N-terminal and C-terminal domain average minimized structures respectively. As can be seen, most of the backbone atoms in the N and C-terminal domains exhibits good structural convergence. The atomic rms distribution about the mean coordinate positions for residues 10 to 80 is 0.66 ± 0.17 Å for backbone atoms, and 1.07 ± 0.14 Å for all heavy atoms, and for residues 98 to 155 is 0.69 ± 0.18 Å for backbone atoms, and 1.17 ± 0.16 Å for all heavy atoms. The structure of the backbone and the hydrophobic core sidechain residues are therefore well defined by the NMR data except for the first four residues of the N-terminal domain, the last five residues of the C-terminal domain, and the linker region between the two domains (residues 85 to 94). ^{15}N-T_2 NMR relaxation data (Chapter V) has revealed that residues 86 to 88, as well as the last two residues at the C-terminus are highly flexible indicating that these regions of the protein experience conformational diversity. As a result of the increased mobility of residues 86 to 88, there is significant structural heterogeneity resulting in a spatial relationship between the two domains which is ill-defined (Figure VII.2C).

Table VII.2. Rms deviations from idealized values:

Bonds (A)	0.004 ± 0.000
Angles (degrees)	0.403 ± 0.011
Impropers (degrees)	0.308 ± 0.011

XPLOR Energies (kcal mol^{-1}):

E_{total}	279.4 ± 23.6
§E_{vdw}	66.4 ± 17.6
$E_{L\text{-}J}$	-650.9 ± 21.4
†E_{cdih}	3.0 ± 0.6
*E_{noe}	40.3 ± 7.1

The values reported are for the ensemble of 23 X-PLOR structures generated from the simulated annealing protocol as described in the X-PLOR 3.1 manual (Brünger, 1992).

*Inter-proton distance information was approximated from 3D ^{15}N-edited NOESY, 3D ^{13}C-edited NOESY, 4D ^{15}N, ^{13}C edited NOESY, and 2D homonuclear NOESY experiments with mixing times of 75 ms and 50 ms (for the 4D experiment) (see Chapter III for more details). In total 2106 distance restraints were applied with a soft square potential and a force constant of 50 kcal mol^{-1} Å^{-2}. Of these restraints, 996 were intra-residue, 505 were sequential, 321 were medium range, and 284 were long range NOE's.

†ϕ dihedral angle restraints were obtained from HMQC-J experiments and ψ dihedral angle restraints were obtained from the $d_{N\alpha}/d_{\alpha N}$ ratio (Gagné et al., 1994; Sykes et al., 1995) In total, 121 ϕ restraints, and 76 ψ restraints were applied with a square well potential and a force constant of 200 kcal mol^{-1} rad^{-2} during the cooling stage.

§The X-PLOR F_{repel} function was used to simulate the vanderWaals interactions with atomic radii set to 0.75 times their CHARMM (Brooks et al., 1983) values. E_{vdw} and E_{total} reflect the use of this approximation. In the final minimization step of simulated annealing, a 6-12 Lennard-Jones potential was applied and the energy is as shown with the variable $E_{L\text{-}J}$.

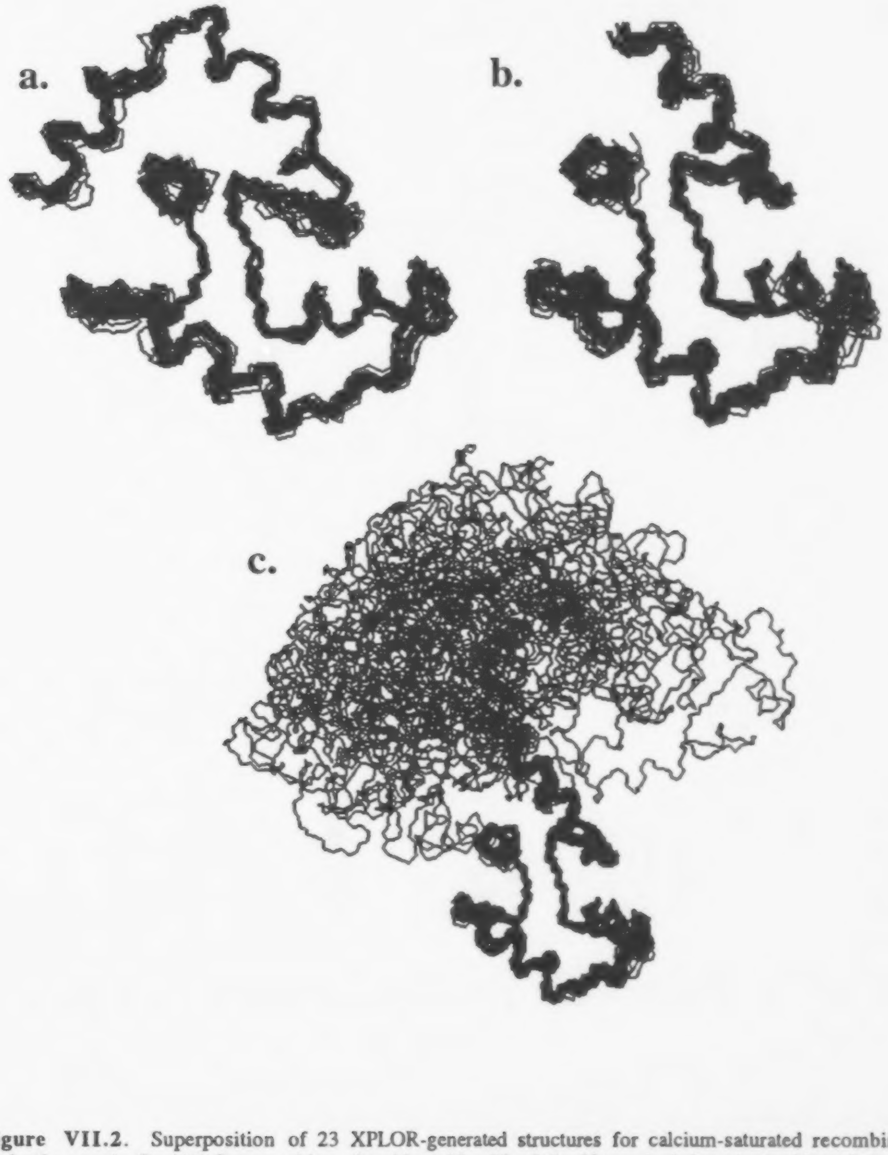


Figure VII.2. Superposition of 23 XPLOR-generated structures for calcium-saturated recombinant chicken skeletal troponin C. (A) Superposition of residues 10 - 80 of the N-terminal domain (residues 1 to 90) onto the mean atomic coordinates. Shown are residues 5 to 83. (B) Superposition of residues 98 to 155 of the C-terminal domain (residues 91 to 162) onto the mean atomic coordinates. Shown are residues 95 to 158. (C) Same as B, but showing residues 5 to 158.

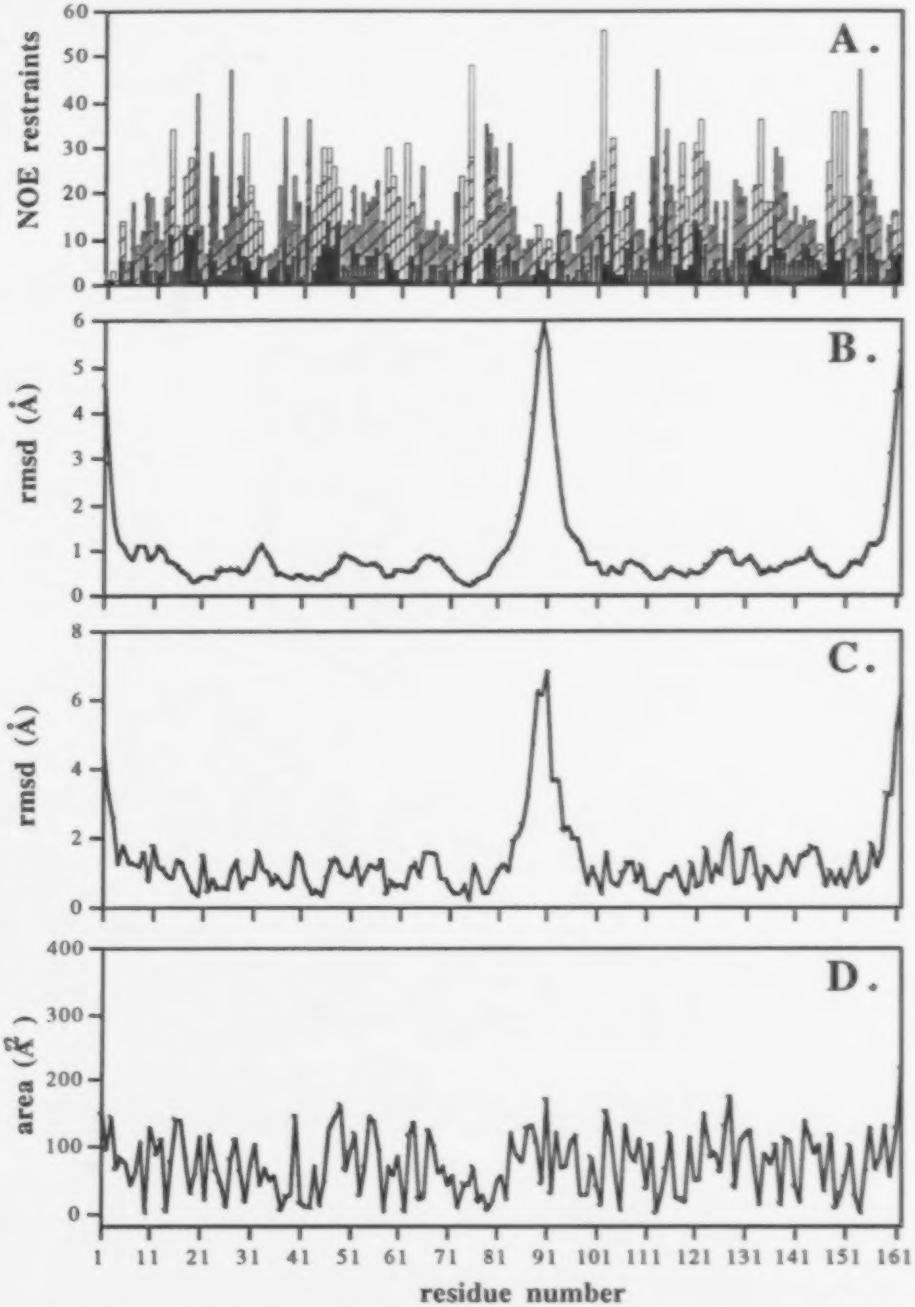


Figure VII.3. (A) Distributions of NOEs that were used in the structure calculations. The height reflects the total number of NOE-derived restraints for each residue. Intra-residue, sequential and medium range, and long range NOE's are indicated by the black, cross-hatched, and white portions of the box. Atomic rms deviations (Å) of the 28 individual structures about the mean structure for (B) backbone atoms (N, Cα, C') and (C) heavy atoms (all atoms except hydrogens). (D) Residue-based solvent-accessible surface area in Å^2.

Figure VII.3 illustrates the compilation of statistics from each domain which have been concatenated to generate statistics for the whole molecule. The number of NOE restraints, the rms deviations from the average structure of the backbone and heavy atoms, along with the variation in surface accessibility on a residue per residue basis are shown. On average, 13 NOE restraints per residue were used in the structure calculations. Areas which lacked NOE restraints (Residues 1 to 4, 33 to 35, 85 to 94, and 158-162) in general were the least well ordered as shown in Figure VII.3 B and C. The RMSD's indicate that the overall structure is very well defined (except for the ends of the molecule and the central region where conformational heterogeneity exists), with the regular secondary structure elements (α-helices and β-sheets) extremely well defined. Residues which have a slightly larger RMSD generally have greater solvent accessible surface areas as shown in Figure VII.3 D.

The convergence of the TnC structures was also analyzed by obtaining the angular order parameter of the ϕ, ψ, and χ^1 torsion angles as a function of residue number according to the method of Hyberts et al. (1992). The angular order parameter is a measure of the deviation of a particular torsion angle. If the angular order parameter has a value of 1, then the angle is identical in all structures. If the angular order parameter has a value of 0, then the angle deviates in a random manner, and thus a disordered structure is inferred. A value of $S = 0.99$ corresponds to a standard deviation of $\pm 7.5°$, a value of $S = 0.95$ corresponds to a standard deviation of $\pm 17°$, and a value of $S = 0.9$ corresponds to a standard deviation of $\pm 24°$. Thus, the order parameter is related to the standard deviation in a logarithmic manner (Hyberts et al., 1992). It is important to note that, for residues that have symmetrically branched sidechains such as Asp, Glu, Leu, Phe, Tyr, and Val, low values of the angular order parameter may be obtained but may not always result from poor convergence. Instead, a 180° rotation of the side-chain atoms may produce two equally favorable yet out-of-phase populations with a near zero angular order parameter. Analysis of the side-chain superpositions allows one to easily distinguish between random sampling of angle space and two equally populated 180° out-of-phase torsional angles (Meadows et al., 1993).

The order parameters shown in Figure VII.4 indicate that the overall structure, in terms of torsion angles, is very well defined. Angular order parameters for the ϕ and ψ backbone dihedrals had mean values of 0.98 ± 0.1, and 0.97 ± 0.1 (for residues 5 to 158), respectively indicating very good convergence for these torsional angles. Residues with low angular order parameters (< 0.90) are residues at the beginning and ends of the molecule (residues 1, 2, 3, 4, 5, 158, 159, 160), as well as a few others (33, 34, 35, 71, 87, 90, 92, 95, 119, 127, 128). Residues at the ends of the molecule tend to be disordered, and thus low angular order parameters for these residues are to be expected. Residues 87, 90, 92, and 95 are present in the region of TnC associated with conformational heterogeneity. The low angular order parameters in this region may be because of too few NMR restraints. The low angular parameters obtained for residues 33, 34, 35, 71, 119, 127, and 128, which are all glycines (except for residue 128), are most likely due to the lack of experimental restraints (see Figure VII.3).

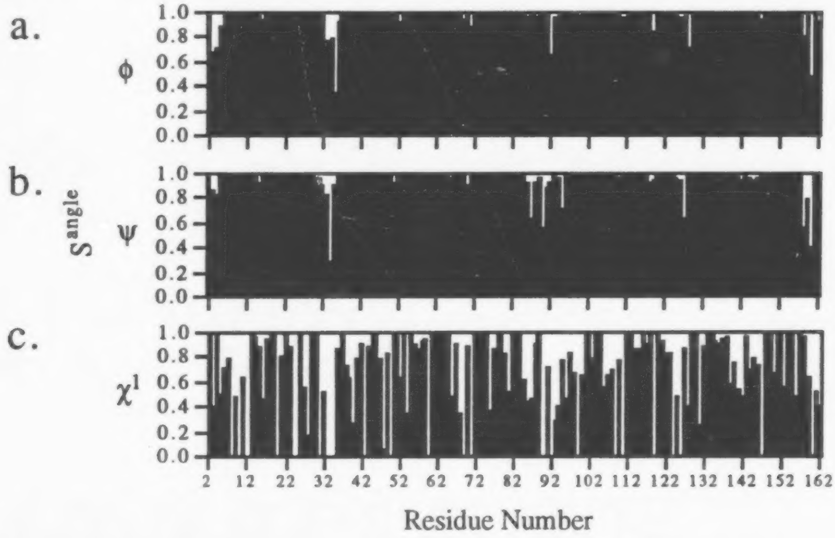


Figure VII.4. Order parameters for (a) ϕ, (b) ψ, and (c) χ^1 angles for the 23 structures of calcium-saturated skeletal TnC as a function of residue number.

Figure VII.5 illustrates the individual superimposed helices N, A, B, C, D, E, F, G, and H, as well as the β-sheet regions in the N and C-terminal domains together with some of the well-defined sidechain residues. As is evident from Figures VII.3 to VII.5, the high definition of the structures is not confined solely to the backbone. Many of the sidechains exhibit atomic rms deviations with respect to the mean of less than 1 Å, and order parameters close to 1. Almost all of the ill-defined sidechains, however, exhibit a larger surface accessibility in the minimized mean structure when compared to the well-defined sidechains indicating greater solvent exposure for these sidechains. Those sidechains which have a χ^1 angular order parameter of less than 0.8 are M3, D5, Q6, Q7, E9, R11, E16, D27, M28, D32, S38, T39, K40, E41, R47, M48, N52, T54, D66, D68, E76, M81, Q85, M86, K87, K91, K93, S94, E95, E96, L98, N100, R103, D106, K107, N108, D110, T125, H128, E131, K139, D140, S141, D142, N144, N145, D146, D150, E153, E156, E159, V161, and Q162. As can be observed, most of the residues with ill-defined sidechains are charged or polar. M3 and V161 are hydrophobic residues present at the ends of the molecule and thus it is likely that their sidechains exhibit conformational heterogeneity. Two of the methionines (28 and 48) exhibit greater solvent accessible surface areas (110 and 140.8 Å^2 respectively). Methionines 81 and 86, and leucine 98, however, have somewhat buried sidechains (solvent accessible surface areas of 47.4, 78.3, and 27.5 Å^2 respectively), and χ^1 angular order parameters of approximately 0.55.

Figure VII.6 shows a Ramachandran plot of the ϕ and ψ angles for the average structure, as well as all 23 structures. For the average structure, all backbone torsion angles of non-glycine residues fall in the allowed regions of the Ramachandran ϕ,ψ plot (glycine residues are indicated by the triangular markers, and all other residues are indicated by the square markers). The two square markers present in the allowed positive phi region are alanine 109, and asparagine 145 which will be discussed below. For all 23 structures, 89% of the ϕ, ψ angles fall within the most favored region of the Ramachandran plot (marked as A, B, L, and P), 11% fall within the additionally allowed regions (marked as a, b, and l), and 0.3% fall within the generously allowed regions (marked as ~a, ~b, ~l, and ~p). Those residues present in the generously allowed regions and disallowed regions are indicated. Out of 3726 residues in the 23 structures, there are only two residues which are within the disallowed region, namely aspartic acid 5, and asparagine 145. Aspartic acid 5 is a residue near the N-terminal region of the protein, and as indicated above, exhibits a lower angular order parameter. Asparagine 145 is a residue in the C-terminal domain calcium binding loop. This residue, and the equivalent residue in calcium binding site III (alanine 109), are involved in a type III Asx turn ((ϕ, ψ) angles of approximately (60°, 30°)) as was observed in the crystal structure for these residues (Herzberg and James, 1985). The equivalent residues in the N-terminal domain are glycines (33 and 69). The angular order parameter for asparagine 145 is > 0.9 for the ϕ and ψ angles, and therefore, in general, this residue is well defined, however for one structure a ψ angle of 135° was found. This value for the ψ angle is within the error limits put on the residue for the angle (120° ± 100). The backbone RMSD for residues in the calcium-binding loop regions are generally slightly higher than for other, well-defined regions (in terms of secondary structure) of the molecule which could account for the deviation in one of the structures.

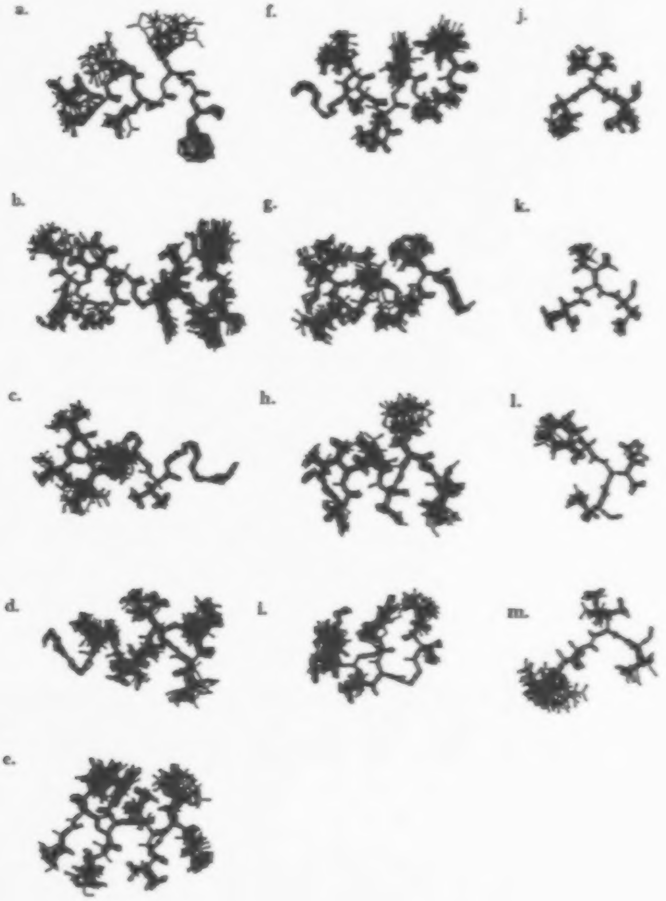


Figure VII.5. Superposition of the secondary structure elements of TnC with some sidechains. (a) N-helix (residues 5 to 13) illustrating all sidechain residues (backbone RMSD = 0.17 ± .08); (b) Helix A (residues 16 to 30) illustrating the sidechains of residues 18, 19, 20, 22, 24, 25, 26, 27, 29, and 30 (backbone RMSD = 0.25 ± .08); (c) Helix B (residues 38 to 49) illustrating the sidechains of residues 38, 39, 41, 42, and 45 (backbone RMSD = 0.21 ± .07); (d) Helix C (residues 55 to 65) illustrating the sidechains of residues 58, 60, 61, 62, 64, and 65 (backbone RMSD = 0.26 ± .12); (e) Helix D (residues 74 to 84) illustrating the sidechains of residues 74, 75, 77, 78, 79, 80, 82, and 83 (backbone RMSD = 0.17 ± .06); (f) Helix E (residues 95 to 105) illustrating the sidechains of residues 98, 99, 100, 102, 104, and 105 (backbone RMSD = 0.25 ± .06); (g) Helix F (residues 114 to 124) illustrating the sidechains of residues 115, 116, 118, 121, and 122 (backbone RMSD = 0.26 ± .13); (h) Helix G (residues 131 to 141) illustrating the sidechains of residues 133, 134, 135, 136, 138, and 140 (backbone RMSD = 0.24 ± .06); (i) Helix H (residues 150 to 158) illustrating the sidechains of residues 151, 152, 154, 155, and 157 (backbone RMSD = 0.15 ± .05); (j) β-sheet in calcium binding site I (residues 36 to 38) (k) β-sheet in calcium-binding site II (residues 72 to 74) (backbone RMSD for antiparallel sheet = 0.20 ± .09) (l) β-sheet in calcium binding site III (residues 112 to 114) (m) β-sheet in calcium binding site IV (residues 148 to 150) (backbone RMSD for antiparallel sheet = 0.13 ± .04).

Description of the Structure: The structure of calcium saturated TnC reveals a protein consisting of two domains with a total of nine helices (N (5 - 13), A (16 - 29), B (39 - 48), C (55 - 64), and D (75 - 84) in the N-terminal domain and E (95 - 105), F (116 - 124), G (131 - 141), and H (151 - 158) in the C-terminal domain) and two antiparallel β-sheet regions (residues 36 to 38 and 72 to 74 forming an antiparallel β-sheet in the N-terminal domain, and residues 112 to 114, and 148 to 150 forming an antiparallel β-sheet in the C-terminal domain) forming four helix-loop-helix calcium binding sites. The four calcium binding loop regions start with a type I turn (Figure VII.7) as evidenced by NOE data (Chapter VI) and ϕ, ψ angles, which consist of residues 30 to 41 (site I), 66 to 77 (site II), 106 to 117 (site III), and 142 to 153 (site IV). Helices A and B as well as C and D in the N-terminal domain and helices E and F as well as G and H in the C-terminal domain form the helices flanking the calcium binding loops (the so-called Ca^{2+} binding EF-hands). The N-helix (residues 5 - 13) wraps around helix D, making several contacts between the side chain residues of both helices. Residues 85 to 94 are ill-defined by the present data and appear to be disordered. Support for a disordered central region of TnC is the observation of differential linewidths for the two domains, and increased mobility of residues in the central region as evidenced by ^{15}N-T_2 data (Chapter V).

Figure VII.8 illustrates stereo views of the ensemble of NMR-derived structures with the well-defined hydrophobic core sidechains appropriately colored (hydrophobic residues (alanine, valine, leucine, and isoleucine) are green, phenylalanine residues are brown, methionine residues are yellow). The figure illustrates five phenylalanine residues present in the hydrophobic pocket of the N-terminal domain (residues 22, 26, 29, 75, and 78), and four in the C-terminal domain (residues 102, 105, 151, and 154). Shown as well are several isoleucine, leucine and valine residues. The core of the hydrophobic pocket arises from residues primarily from the amphipathic helices comprising the EF-hand calcium-binding loops as well as residues from the calcium binding loop itself. As can be observed from the figure, the hydrophobic pocket present in the N-terminal domain appears to have a larger surface area than the hydrophobic pocket of the C-terminal domain.

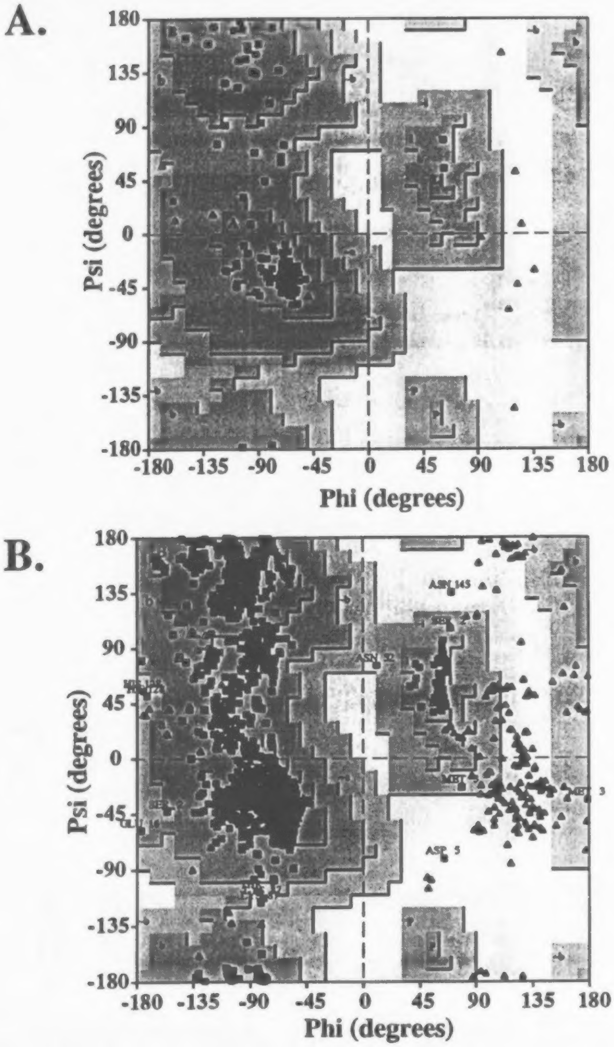


Figure VII.6. Ramachandran ϕ, ψ plot for (A) the average structures of the N and C-terminal domains, and (B) all 23 structures. The plot was generated using the program PROCHECK.

The larger exposed hydrophobic surface area of the N-terminal domain as compared to the C-terminal domain may also be observed in Figure VII.9B, and C where the structures of the minimized average N-terminal and C-terminal domains are illustrated in CPK format. All atoms from hydrophobic residues are shown in green, whereas all other atoms are shown in white. Also shown in Figure VII.9A is the CPK format of the crystal apo N-terminal domain calcium binding sites. This figure illustrates a substantial opening of the N-terminal domain upon binding of calcium to expose a large hydrophobic area, which appears to be much larger than that observed for the C-terminal domain. This large exposed hydrophobic area is most likely the cause of the calcium-induced dimerization characterized in Chapter V which involves the hydrophobic patch of one TnC N-terminal domain interacting with the N-terminal domain hydrophobic patch of a symmetrically related TnC molecule.

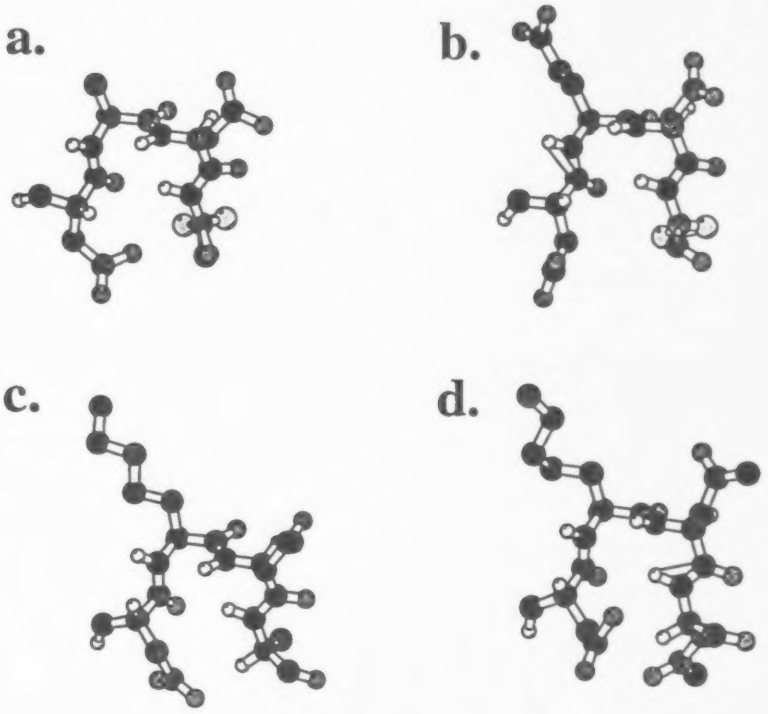
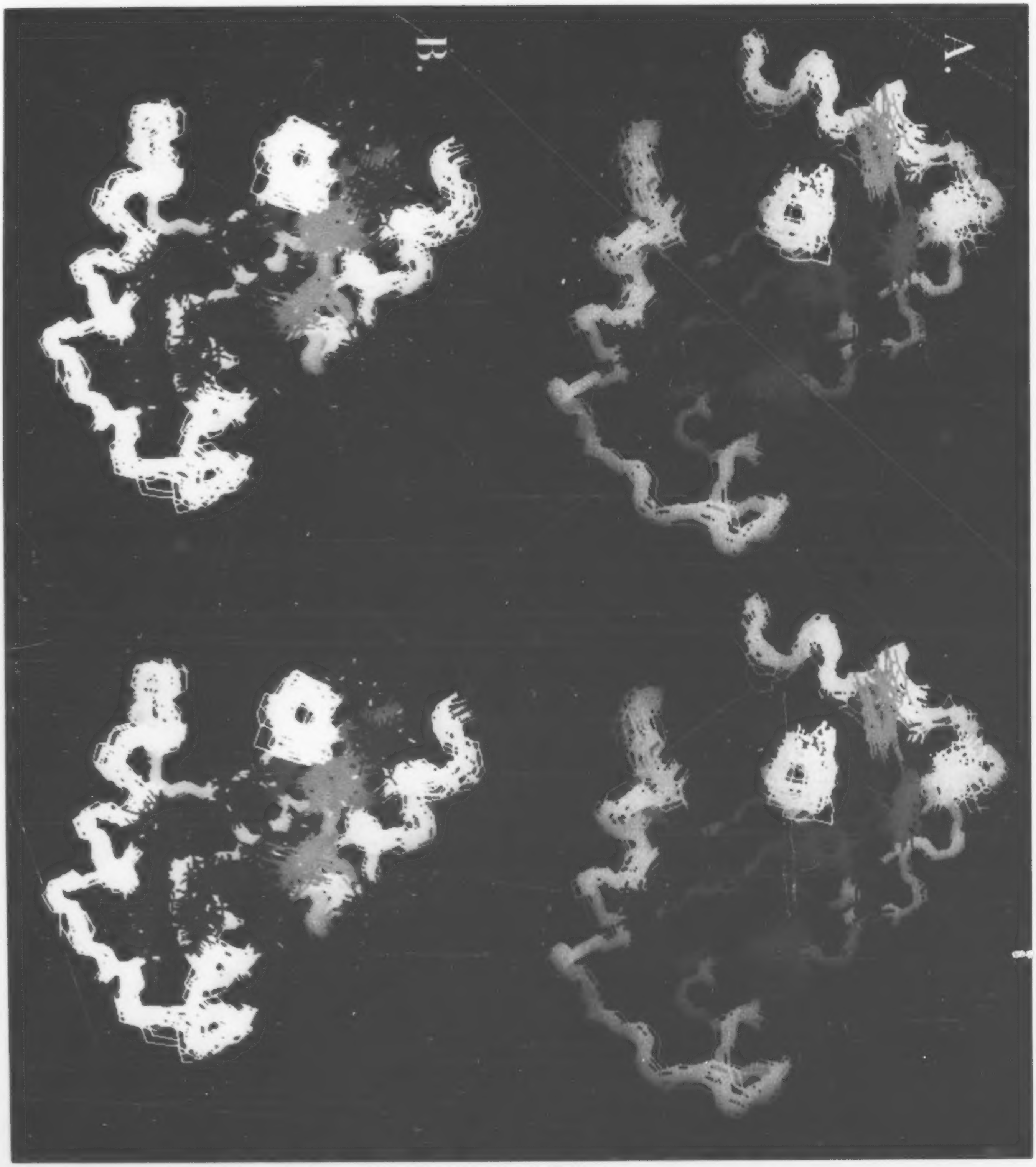
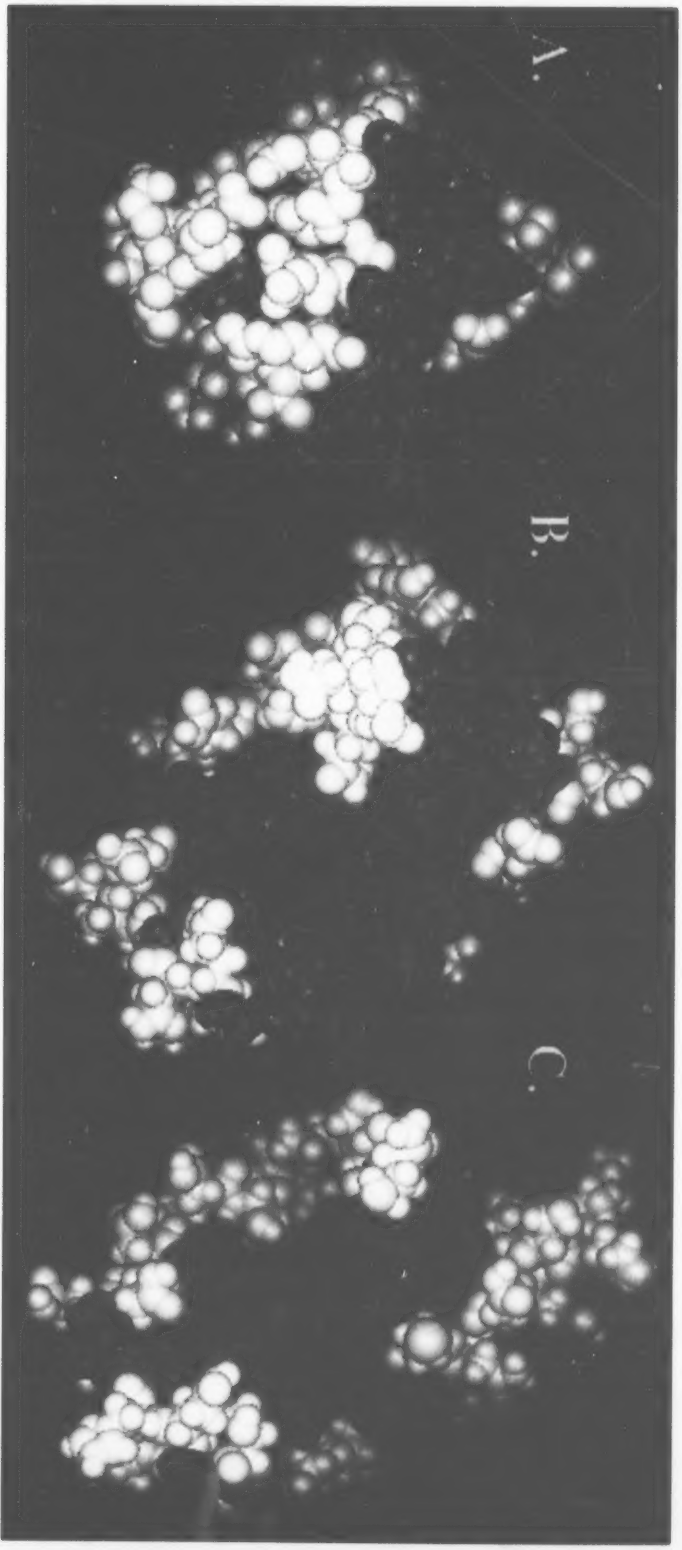


Figure VII.7. The four type I turn conformations found at the beginning of each of the calcium binding loops shown from the minimized mean structure of TnC. (a) calcium binding loop I (residues 30 to 33), (b) calcium binding loop II (residues 66 to 69), (c) calcium binding loop III (residues 106 to 109), (d) calcium binding loop IV (residues 142 to 145). This figure was prepared using MOLSCRIPT (Kraulis, 1991)

Figure VII.8: (Page 171) Stereo views of the backbone and selected sidechain residues of 23 superimposed NMR-derived structures of (A) the N-terminal domain of TnC, and (B) the C-terminal domain of TnC. Details of the structure calculation may be found in Chapter III. This figure was prepared using BIOSYM INSIGHT II software.

Figure VII.9. (Page 172) CPK illustrations of (A) X-ray crystal structure of the apo N-terminal domain of turkey TnC (from Herzberg and James, 1988), (B) Average minimized NMR-derived structure of the calcium-saturated N-terminal domain of recombinant chicken skeletal TnC, and (C) Average minimized NMR-derived structure of the calcium-saturated C-terminal domain of recombinant chicken skeletal TnC. Shown in green are all hydrophobic sidechain atoms, and in white are all other atoms. This figure was prepared using BIOSYM INSIGHT II software.


A.
B.


A.
B.
C.

Comparison of the NMR-derived structure of calcium-saturated TnC to other structures: EF-hand calcium binding domains are present in a variety of proteins with differing functions (see Chapter I.2). One of the most closely related proteins to TnC, in terms of structure, is calmodulin. The elements of secondary structure of the two proteins have been shown, upon alignment of the calcium binding loops, to be homologous to one another (Chapter VI), and to the half-saturated x-ray crystallographic form of TnC (Chapter VI). There have been many studies of both calmodulin and TnC in terms of structure. Until now, however, the calcium-saturated structure of TnC had not been solved. Now that this structure is complete, it appears that both TnC and calmodulin share more similarities than was originally thought. Figure VII.10 illustrates ribbon diagrams of the NMR-derived calcium saturated structure of TnC in comparison with half saturated TnC (x-ray crystal structure) (Herzberg and James, 1988), the model structure based on the C-terminal domain of TnC (Herzberg and James, 1986), the structure of calcium-saturated NTnC (Gagné et al., 1995), and calcium-saturated calmodulin (Chattopadhyaya et al., 1994). The RMSD for backbone atoms comprising residues 98 to 155 is 1.22 Å between the average minimized NMR structure and the crystal structure of TnC, and 1.67 Å between residues 85 to 142 of calmodulin and 98 to 155 of the average minimized structure of TnC. The NMR-derived structure of TnC therefore most closely resembles the crystal structure of TnC. Several studies confirm that the C-terminal domain of calmodulin behaves differently from the C-terminal domain of TnC. One study of proteolytic fragments of calmodulin and TnC revealed that the C-terminal domain of calmodulin, but not TnC, could interact with a hydrophobic interaction chromatography column (Vogel et al., 1983).

The conformation of the N-terminal domain of TnC is markedly different from the C-terminal domain, the x-ray structure, or the model structure, but is similar to the N-terminal domain of calmodulin and the calcium-saturated structure of NTnC. The RMSD for backbone atoms comprising residues 5 to 83 is 5.45 Å between the apo x-ray structure of TnC and the average minimized calcium-saturated structure of TnC, and 1.06 Å upon alignment of only residues 5 to 30 and 75 to 83. Therefore, the major difference between the x-ray structure of half saturated TnC and the NMR structure of calcium-saturated TnC is as was predicted by Herzberg et al. (1986), which is the movement of helices B and C away from helices N, A, and D. The RMSD for backbone atoms comprising residues 5 to 83 of TnC and NTnC is 1.28 Å, and between residues 6 to 73 of calmodulin and 16 to 83 of TnC is 1.25 Å. These RMSD values suggest that the structure of the isolated N-terminal domain of TnC in its dimeric calcium-saturated form, and the structure of the N-terminal domain of calcium-saturated calmodulin bear a striking resemblance to the N-terminal domain of calcium-saturated TnC. A comparison of the structure of the N-terminal domain of calcium-saturated TnC with that of the model of calcium-saturated TnC reveals an RMSD of 2.25 Å suggesting that although the model structure, in general, predicts what the global change is when TnC binds calcium, the actual structure is somewhat different. The model predicts that the change should be similar to the calcium-saturated structure of the C-terminal domain of TnC; however the actual structure of the calcium-saturated N-terminal domain of TnC is more similar to the calcium-saturated N-terminal domain of calmodulin than to the calcium-saturated C-terminal domain of TnC. Pearlstone et al. (1991b) also suggested that the actual structure may be somewhat different than what was predicted.

Table VII.3. Comparison of inter-helical angles.*

N-domain

Coordinates	B to A	C to N	C to B	D to A	D to B	D to C
Crystal	**131.7**	**132.9**	131.5	117.8	47.8	**144.7**
Model	**98.6**	**97.4**	127.1	113.1	34.2	**111.3**
Calmodulin	**85.1**	**n/a**	113.3	107.7	40.3	**86.8**
Ave-NMR	**79.2**	**87.2**	117.6	108.8	57.0	**80.8**

C-domain

Coordinates	F to E	G to E	G to F	H to E	H to F	H to G
Crystal	107.2	128.8	123.2	118.6	35.2	108.2
Calmodulin	101.3	146.6	112.1	119.4	40.7	87.9
Ave-NMR	97.8	135.0	127.0	112.5	25.6	108.0

*As determined by the program "bestfits" and "interhx" provided in the Ribbons 2.0 package (Carson, 1987).

Table VII.3 is a summary of the inter-helical angles comparing calcium saturated TnC, calcium saturated calmodulin (Babu et al., 1988; Chattopadhyaya et al., 1994), half-saturated TnC (x-ray crystal structure) (Herzberg and James, 1988; Satyshur et al., 1988, 1994) and the Herzberg-James model for the N-domain of calcium-saturated TnC (Herzberg et al., 1986). The conformation of the C-domain resembles both the x-ray crystal structure as well as calmodulin in terms of the interhelical angles, except for the H to G interhelical angle. In calmodulin, the H to G interhelical angle indicates a slightly more open C-terminal domain than for TnC thus accounting for the higher RMSD between calmodulin and TnC, and accounting for the apparent more open structure of the C-terminal domain of calmodulin versus TnC (Vogel et al., 1983).

The conformation of the N-terminal calcium-saturated domain of TnC resembles that of calmodulin in terms of interhelical angles, but appears to be more open upon comparison with the model structure (a decrease in the interhelical angle of B to A, C to N or D to C indicates a more open structure), and very much more open upon comparison with the crystal apo N-terminal domain. The interhelical angles presented here for calcium-saturated TnC are similar to those obtained for the NTnC dimer (Gagné et al., 1994). This indicates that the structures of the calcium-saturated NTnC dimer and the TnC monomer are similar. These results suggest that dimerization has little effect on the tertiary structure of TnC, and that the C-terminal domain of TnC has no effect on the structure of the N-terminal domain of TnC. These results also indicate that studies involving fragments of the N-terminal and C-terminal domains of TnC are good approximations of the whole molecule.

It is clear from these data that 15% v:v TFE has little effect on tertiary structure thus reinforcing the use of TFE as a general perturbant of quaternary structure. As well, dimerization does not appear to have a major effect on the tertiary structure of TnC, as the RMSD between NTnC and the N-terminal domain of TnC is low. Both structures therefore validate one another and most likely represent the structure of the N-terminal domain of TnC when bound to TnI. Further, since TFE is a small molecule, the binding of some TFE to TnC would result in very little increase in molecular weight of TnC, in contrast to the detergent CHAPS, making its general use as a denaturant of quaternary structure applicable.

Shown here, for the first time, is the structure of calcium saturated TnC which demonstrates two aspects of its function making it different from most other similar calcium binding proteins. First, upon calcium binding, the N-domain hydrophobic pocket opens up to produce a hydrophobic area which is more exposed than in the absence of calcium. This large exposed hydrophobic area is the reason for dimerization of TnC and is what would enhance the interaction between the N-terminal domain of TnC, and the C-terminal domain of troponin I in the muscle filament. Second, the flexible linker between the two domains allows TnC to explore a variety of orientations with respect to the C-terminal domain of TnC while the C-terminal domain remains firmly bound to the thin filament. The effect of the flexible linker could possibly be to decrease the reaction time when calcium is released into the muscle cell resulting in faster contraction. Another effect is to allow the two domains to adopt any orientation with respect to one another. Indeed, several crosslinking studies have indicated that when the inhibitory component is bound to TnC, crosslinks may be made to helix C of the N-terminal domain (Leszyk et al., 1990; Wang et al., 1990; Kobayashi et al., 1991). One model of the TnC/TnI complex suggests that TnC is elongated (Olah and Trewhella, 1994). Our results neither prove nor disprove this model. From all the results presented thus far on the interaction of these two proteins, it is clear that some sort of flexibility must be present in the central helix to allow the two domains to reorient with respect to one another so that TnC may optimally interact with TnI.

The results presented here suggest that there may be a general mechanism for the activation of proteins which are members of the Troponin C family. This group of proteins includes calmodulin and TnC, proteins which need calcium in order to interact with their target proteins in contrast to proteins which are merely calcium buffers such as parvalbumin, S100 or calbindin. The first requirement of these proteins is that in the absence of calcium, the hydrophobic patch is not solvent accessible. Secondly, upon calcium binding, a large hydrophobic patch which is accessible to solvent should form to allow the protein to interact with its target. Thirdly, the interhelical connector has to be flexible enough to allow the two domains to adopt any orientation with respect to one another which would allow both domains to interact with their targets in an optimal manner.

Calmodulin and TnC are different in two respects which is most likely related to the function of these two proteins. First, TnC contains an N-terminal α-helical arm which has been proposed to moderate the destabilization of the central helix making it less flexible than in calmodulin (Ding et al., 1994). Indeed, if TnC should adopt an elongated conformation in complex with other proteins of the thin filament, a more flexible linker could make complexation more difficult. Second, the C-terminal domain of TnC appears to be different from calmodulin. In the absence of calcium, the C-terminal domain of calmodulin remains structured (Finn et al., 1993); however the C-terminal domain of TnC is not structured. The C-terminal domain of TnC can bind magnesium, and thus when calcium is removed from the muscle cell upon relaxation, the C-terminal domain remains in a similar conformation as when calcium is bound. This would be important in the muscle cell so that TnC would remain firmly anchored to the thin filament during relaxation or contraction. Calmodulin, on the other hand, is not normally permanently bound to any other protein, and thus removal of calcium from the C-terminal sites would ensure that in the absence of calcium, calmodulin does not interact with its targets.

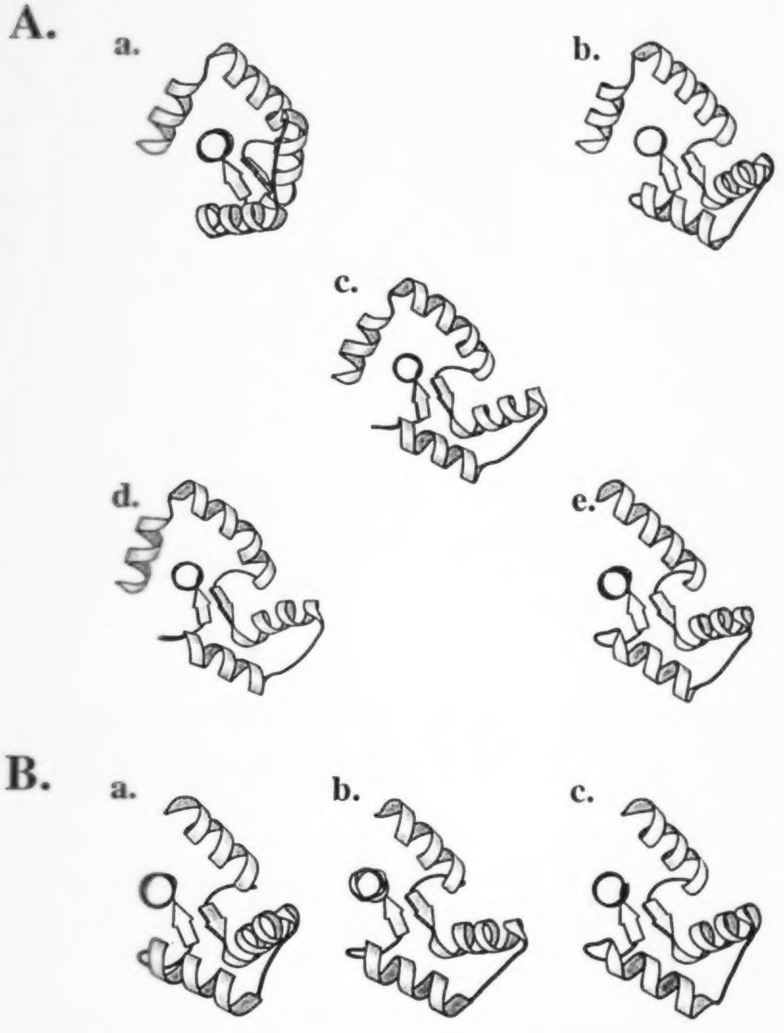


Figure VII.10: Ribbon plots depicting the (A) N-terminal domain of (a) the half-saturated x-ray structure (residues 5-83) (Herzberg and James, 1988), (b) the Herzberg-Moult-James model structure of TnC (residues 5-83) (Herzberg et al., 1986), (c) the average calcium saturated NMR structure (residues 5-83) (d) the average calcium-saturated NTnC structure (residues 5-83) (Gagné et al., 1995); (e) calcium-saturated calmodulin (residues 16-73) (Chattopadhyaya et al., 1994), and (B) the C-terminal domain of (a) the half-saturated x-ray structure (residues 98-155) (Herzberg and James, 1988), (b) the average calcium saturated NMR structure (residues 98-155), and (c) calcium saturated calmodulin (residues 85-142) (Chattopadhyaya et al., 1994). The ribbons were generated using MOLSCRIPT (Kraulis, 1991).

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Chapter VIII:

A ^{1}H NMR Study of a Ternary Peptide Complex that Mimics the Interaction between Troponin C and Troponin I.[1]

*M*uscle contraction involves multiple protein interactions. The thick filament protein myosin interacts with the thin filament proteins actin, tropomyosin and troponin (which is composed of three subunits, troponin C, troponin I, and troponin T). Troponin C (TnC) binds calcium, troponin I (TnI) inhibits the magnesium dependent ATPase activity of actomyosin (Hartshorne and Mueller, 1968; Schaub and Perry, 1969; Greaser and Gergely, 1971; Perry et al., 1972) through interactions with both actin and tropomyosin-actin (Potter and Gergely, 1974; Hitchcock, 1975) and troponin T (TnT) binds to tropomyosin. The inhibition of the actomyosin ATPase is neutralized when calcium-saturated TnC forms a complex with TnI (Perry et al., 1972; Weeks and Perry, 1978; Chong et al., 1983). This calcium dependent interaction between TnC and TnI is one of the key processes in the regulation of contraction in skeletal muscle and thus is important to the understanding of the mechanism of muscle contraction on a molecular level (for recent reviews, see Zot and Potter, 1987; Leavis and Gergely, 1984).

Studies indicate that residues 1-21 and 96-116 of TnI interact with TnC (Syska et al., 1976; Moir et al., 1974; Cole and Perry, 1975). Residues 104-115 of TnI (Gly-Lys-Phe-Lys-Arg-Pro-Pro-Lys-Arg-Arg-Val-Arg) comprise the minimum sequence necessary for inhibition of actomyosin ATPase activity (Talbot and Hodges, 1979; Cachia et al., 1983,1985; Van Eyk and Hodges, 1991). It was shown that this peptide is able to substitute for TnI in skinned cardiac muscle fibers by binding to actin in the absence of calcium, to inhibit force development, and to TnC in the presence of calcium, to release the inhibition (Van Eyk et al., 1993). This TnI peptide is extremely basic, with four arginines and two lysines within a twelve residue sequence. Alternating with these basic residues are hydrophobic residues. Van Eyk et al. (1988, 1991) have evaluated the contribution of each amino acid residue of the TnI inhibitory region (104-115) and have determined that the most important residues for binding and inhibitory activity were F106, R108, L111, R113, V114 and R115. The structure of the TnI peptide bound to calcium-saturated s-TnC, derived from two dimensional transferred nuclear Overhauser effect ^{1}H NMR spectroscopy, reveals an amphiphilic helix-like structure, distorted in the center by two proline residues (Campbell and Sykes, 1989, 1991a, 1991b). The central bend in the peptide functions to bring the residues on the hydrophobic face into closer proximity with each other to form a small hydrophobic pocket with the hydrophilic, basic residues extending off the opposite face of the peptide.

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[1] A version of this chapter has been published. Slupsky, C.M., Shaw, G.S., Campbell, A.P., and Sykes, B.D. 1992. Protein Science 1: 1595-1603.

Although the inhibitory region of TnI which interacts with s-TnC has been defined, the region on s-TnC which interacts with the inhibitory region of TnI is not clearly defined. The crystal structure of s-TnC (Herzberg and James, 1985, 1988; Satyshur et al., 1988; Sundaralingam et al., 1985) reveals a dumbbell shaped molecule with two globular domains connected by a nine turn α-helix. Each domain contains two calcium binding sites of the helix-loop-helix structural motif. NMR studies of tryptic fragments of TnC indicate that both halves of the molecule retain a structure in the apo and calcium-saturated forms which resemble the structure of the intact protein (Drakenberg et al., 1987). Recently, it has been shown that synthetic peptides representing the calcium binding site III (SCIII) or site IV (SCIV) of TnC, in the presence of calcium, assemble to form symmetric dimers which are structurally very similar to the C-terminal domain of TnC (Shaw et al., 1990, 1991; Kay et al., 1991). Further, when equimolar amounts of SCIII and SCIV are mixed in the presence of calcium, SCIII·SCIV heterodimers are stoichiometrically and preferentially formed which even more closely mimic the C-terminal domain of s-TnC in structure (Shaw et al., 1991a, 1991b).

Several studies indicate that the TnI peptide interacts with the C-terminal domain of TnC (Cachia et al., 1983; Drabikowski et al., 1985; Lan et al., 1989; Leszyk et al, 1987, 1988; Weeks and Perry, 1978; Grabarek et al., 1981; Ngai et al., 1994; Howarth et al., 1995). In the present study we use ^{1}H NMR spectroscopy to compare the binding of TnIp to the synthetic noncovalent heterodimer comprised of site III and site IV peptides with the binding of TnIp to calcium-saturated s-TnC.

Results and Discussion

TnIp has been shown to interact with the C-terminal domain of TnC (Weeks and Perry, 1978; Grabarek et al., 1981; Leavis et al., 1978; Chong and Hodges, 1981; Wang and Cheung, 1984; Leszyk et al., 1987; Lan et al., 1989; Tao et al., 1986, 1989)). Here, we present a ^{1}H NMR comparison of TnIp binding to s-TnC and TnIp binding to an SCIII/SCIV heterodimer. Identification of specific residues in s-TnC and the SCIII/SCIV heterodimer which are perturbed upon binding of TnIp requires spectral assignment of TnIp, s-TnC and the SCIII/SCIV heterodimer. A complete proton assignment of TnIp has been published (Campbell and Sykes, 1991b). As well, a complete assignment of the calcium-saturated SCIII/SCIV heterodimer has been accomplished (Shaw, G.S. and Sykes, B.D. manuscript in preparation), the partial assignment of which has been published (Shaw et al., 1991a, 1992). The partial assignment of whole turkey skeletal s-TnC was accomplished using standard methods employing 1D ^{1}H NMR calcium titration data and 2D DQF-COSY and NOESY data and will not be presented. Partial proton assignments of the C-terminal domain of rabbit s-TnC and whole rabbit s-TnC have been published (Drabikowski et al., 1985; Tsuda et al., 1988, 1990) and are in agreement with those assignments made of turkey s-TnC. These proteins are highly homologous in sequence and share a similarity in sequence and structure to CaM. The entire ^{1}H spectral assignment of Drosophila recombinant CaM has been accomplished (Ikura et al., 1990). Similarities in the crystal structures of CaM and s-TnC (Babu et al., 1988; Herzberg and James, 1985, 1988; Satyshur et al., 1988; Sundaralingam et al., 1985) suggest that there may be similarities in the ^{1}H NMR spectra of these proteins. The chemical shifts found for some of the resonances in s-TnC are very similar to some of the chemical shifts found in CaM (Ikura et al., 1990). Figure VIII.1 illustrates the partial sequences of turkey s-TnC, SCIII/SCIV, rabbit s-TnC and Drosophila recombinant CaM.

Comparison of heterodimer and s-TnC spectra. Figure VIII.2 illustrates a comparison of the aromatic region of the 600 MHz ^{1}H NOESY spectra of s-TnC and the SCIII/SCIV heterodimer. There are a total of five aromatic residues in the C-terminal domain of turkey s-TnC: F102, F105, F112, F151 and F154. The F105 protons do not exhibit much spectral dispersion and therefore are not indicated in either spectrum. The striking similarity of the NOESY spectra in Figure VIII.2 indicates that the C-terminal domain of s-TnC and the SCIII/SCIV heterodimer form similar structures in solution. Three of the residues (F102, F151 and F154) form an aromatic cluster in the x-ray structures (Sundaralingam et al., 1985; Satyshur et al., 1988; Herzberg and James, 1985,

1988) and Figure VIII.2 illustrates NOESY connectivities between these residues. F102 makes strong contacts to F151 and F154. As well, contacts may be seen between F151 and F154. Figure VIII.3 shows a comparison of the amide NH and αCH to aliphatic regions of the NOESY spectra of s-TnC (A) (the amide and α regions in D_2O) and the SCIII/SCIV heterodimer (B) (the amide region in H_2O and the α region in D_2O). The amide hydrogen of I113 of s-TnC is involved in the antiparallel β-sheet hydrogen bonding to I149 carbonyl oxygen and therefore exhibits slow exchange with the solvent (allowing us to observe the amide resonance on a 2-D NOESY spectrum in D_2O) as well as an extreme downfield shift. The amide hydrogen of I113 of the heterodimer exhibits faster exchange with the solvent (and is therefore not observable on a 2-D NOESY spectrum in D_2O) but still exhibits the extreme downfield shift. This residue is analogous to I100 of CaM which also exhibits slow exchange behavior in D_2O (Ikura et al., 1991). Similar NOE's are observed for s-TnC and the SCIII/SCIV heterodimer in this region. A strong

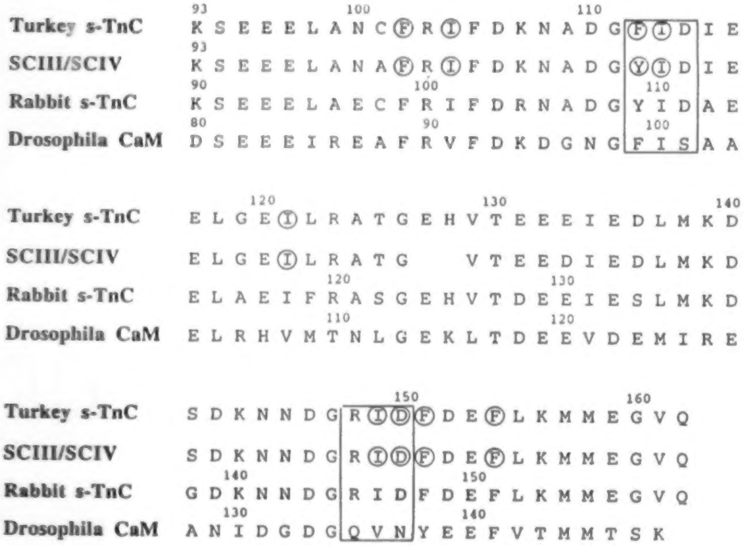
93 100 110
Turkey s-TnC K S E E E L A N C F R I F D K N A D G F I D I E
93
SCIII/SCIV K S E E E L A N A F R I F D K N A D G Y I D I E
90 100 110
Rabbit s-TnC K S E E E L A E C F R I F D R N A D G Y I D A E
80 90 100
Drosophila CaM D S E E E I R E A F R V F D K D G N G F I S A A
120 130 140
Turkey s-TnC E L G E I L R A T G E H V T E E E I E D L M K D
SCIII/SCIV E L G E I L R A T G V T E E D I E D L M K D
120 130
Rabbit s-TnC E L A E I F R A S G E H V T D E E I E S L M K D
110 120
Drosophila CaM E L R H V M T N L G E K L T D E E V D E M I R E
150 160
Turkey s-TnC S D K N N D G R I D F D E F L K M M E G V Q
SCIII/SCIV S D K N N D G R I D F D E F L K M M E G V Q
140 150
Rabbit s-TnC G D K N N D G R I D F D E F L K M M E G V Q
130 140
Drosophila CaM A N I D G D G Q V N Y E E F V T M M T S K

Figure VIII.1: Partial amino acid sequence alignment of turkey s-TnC (Golosinska et al., 1991; Wilkinson, 1976), SCIII/SCIV (Shaw et al., 1991a) (Ac- (A101)(Y112) TnC (93-126) amide / Ac-TnC (129-162) amide), rabbit s-TnC (Collins et al., 1973) and drosophila recombinant CaM (Ikura et al., 1990). The amino acid numbering is indicated at the top of each sequence. Circled residues are those which are perturbed upon binding of the inhibitory TnI peptide. Boxed residues are those involved in the antiparallel β-sheet formation between calcium binding loops III and IV. For the SCIII/SCIV heterodimer, C101 was changed to A101 to prevent intermolecular crosslinks from occuring. F112 was changed to Y112 to aid in making ^1H NMR assignments. Y112 in the heterodimer is equivalent to Y109 in rabbit s-TnC and therefore is a conservative change.

$d_{\alpha N}$ crosspeak between F112α/Y112α and I113NH, and between I149α and D150NH (data not shown) may be observed as well as a cross-sheet $d_{\alpha\alpha}$ between F112α and D150α in s-TnC (Y112α and D150α in SCIII/SCIV). A d_{NN} between I113NH and I149NH and a weak $d_{\alpha N}$ between I113NH and D150α were also observed. COSY and NOESY connectivities between the NH, αCH, βCH, γCH_2 and δCH_3 protons were observed for I113 for s-TnC and the SCIII/SCIV heterodimer.

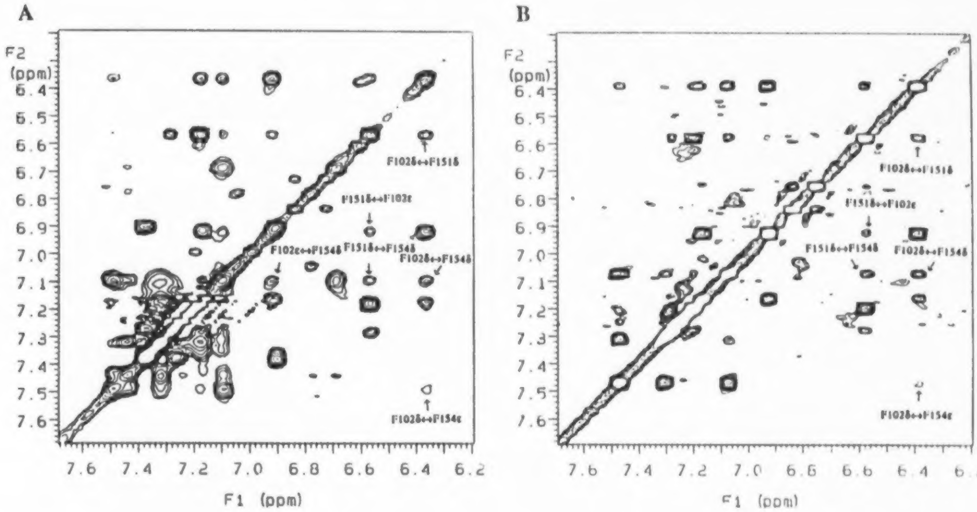

Figure VIII.2: 600 MHz ^{1}H NOESY spectra in D_2O showing NOE connectivities between the phenylalanine rings of residues F102, F151 and F154 for (A) calcium-saturated TnC (2 mM turkey s-TnC, 12 mM $CaCl_2$, 100 mM KCl pD 6.2) and (B) calcium-saturated SCIII/SCIV heterodimer (4 mM SCIII/SCIV, 10mM $CaCl_2$, 50 mM KCl pD 7.2) mM. τ_m = 150 ms for both spectra.

Interactions Between s-TnC and the TnI Peptide. We have used ^{1}H NMR at 600 MHz to follow several residues on turkey s-TnC and the heterodimer which are perturbed upon binding of the inhibitory TnIp. A calcium-saturated s-TnC sample as well as a calcium-saturated heterodimer sample (representing the C-terminal domain of s-TnC) were titrated with TnI peptide. Figure VIII.4a (A and B) shows the aromatic region of the ^{1}H NMR spectra. In both cases, a slight downfield shift is observed for the F102δCH2 and F154δCH2 resonances, and an upfield shift is observed for the F154εCH resonance. The resonances at 6.7 ppm, which arise from the N-terminal domain F75δCH2, F26δCH2

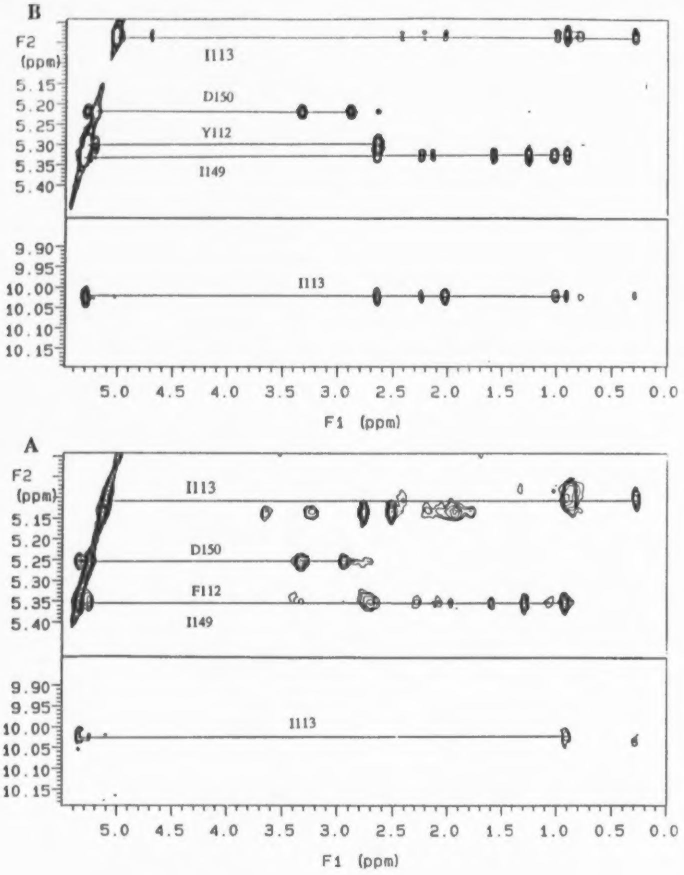

Figure VIII.3: 600 MHz ^{1}H NOESY spectra of (A) calcium-saturated TnC (2 mM turkey s-TnC, 12 mM $CaCl_2$, 100 mM KCl pD 6.2) and (B) calcium-saturated SCIII/SCIV heterodimer (4 mM SCIII/SCIV, 10mM $CaCl_2$, 50 mM KCl (amide region in H_2O pH 7.2 and αCH region in D_2O pD 7.2)) illustrating the amide and αCH regions. τ_m=150 ms for both spectra.

protons (W.A. Findlay and B.D. Sykes, unpublished results), are also observed to shift slightly upon binding of TnIp. As the titration progresses, TnI peptide F106δCH2 and F106εCH2, F106ζCH resonances are observed to emerge at 7.25 and 7.30 ppm, respectively, with increasing peptide concentration. Figure VIII.4b (A and B) shows the α-proton region of the spectra upon binding of TnIp. In both cases, an upfield shift for the I113αCH resonance, a downfield shift for the D150αCH proton, and upfield and downfield shifts for I149αCH and F112(Y112)αCH respectively, were observed. These residues are all situated within the β-sheet region between the two calcium-binding sites. The resonances from D114αCH and R148αCH, which are also present in the β-sheet, are obscured because their resonances are almost coincident with the water resonance. Figure VIII.4c (A and B) shows the upfield shifted methyl region of the ^{1}H NMR spectra during the titration of s-TnC and the heterodimer with the TnI peptide. Marked downfield shifts for the I113δCH_3 and I121γCH_3 resonances and a marked upfield shift for the I104γCH_3 resonance are observed.

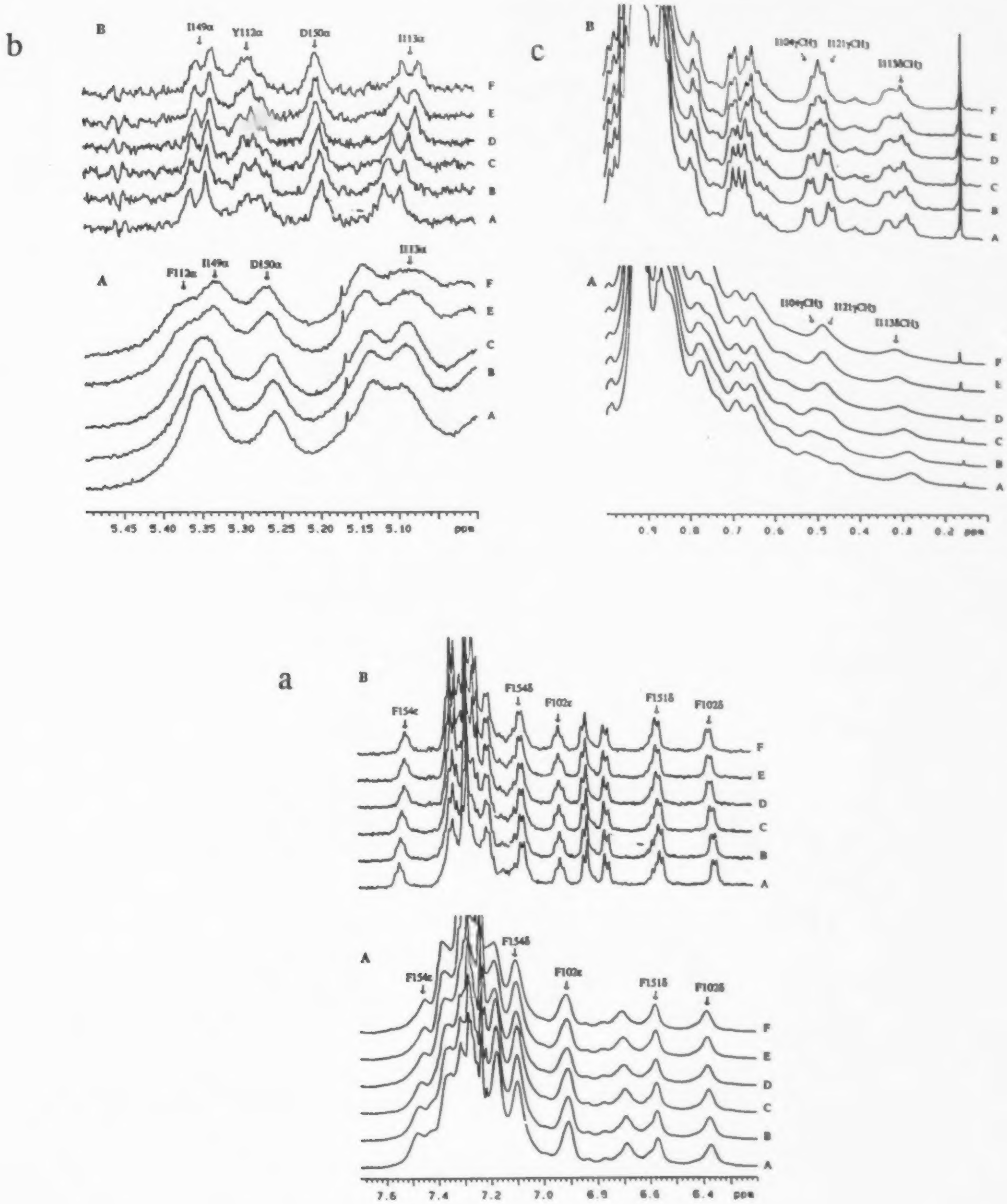

Figure VIII.4: Titration of (A) calcium-saturated TnC and (B) calcium-saturated SCIII/SCIV heterodimer with TnIp. For the TnC spectra, [TnIp]/[TnC] = (A) 0 (B) 0.32 (C) 0.64 (D) 1.29 (E) 1.60 (F) 1.93. Sample was 0.94 mM turkey s-TnC, 12 mM $CaCl_2$, 100 mM KCl, 3 mM DTT in D_2O, pD 6.2, 30°C. For the SCIII/SCIV heterodimer spectra, [TnIp]/[Heterodimer] = (A) 0 (B) 0.30 (C) 0.59 (D) 1.18 (E) 1.48 (F) 2.36. Sample was 152.2 µM heterodimer, 430 µM $CaCl_2$, 50 mM KCl, 30 mM Imidazole-d_4 in D_2O, pD 7.3, 30°C. (a) Aromatic region (b) αCH region (c) methyl region.

The spectral changes presented in Figure VIII.4 indicate that TnIp perturbs the same residues in s-TnC as it does in the SCIII/SCIV heterodimer, and that they are perturbed in the same manner. These residues are from the hydrophobic region formed by the amphipathic helices from calcium-binding sites III and IV, and the β-sheet region between the two calcium-binding loops. The perturbed resonances include the aromatic side chains of F102 and F154, the methyl resonances of I104, I113, and I121, and the backbone αCH resonances of F112(Y112), I113, I149 and D150. This suggests that TnIp binds to the SCIII/SCIV heterodimer and s-TnC in the same way implying, therefore, that the interaction of the TnIp with s-TnC is primarily with the C-terminal domain.

Further evidence for the similarity of the interaction of the TnIp with the heterodimer and s-TnC is obtained from the determination of the stoichiometry and dissociation constant of the TnIp from the NMR data. The change in chemical shift of residues F112α (Y112α), I149α, D150α, I113α, I104γCH$_3$, I121γCH$_3$ and I113δCH$_3$ for the heterodimer were plotted as a function of added [TnIp], and a dissociation constant (K_d) calculated (Figure VIII.5). A dissociation constant was obtained for the heterodimer (1:1 binding with K_d = 200 ± 40 μM for the heterodimer) which is similar to that published for s-TnC using ^{19}F NMR spectroscopy (for a 1:1 complex of TnIp with sTnC K_d = 48 ± 18 μM (Campbell et al., 1991)). The slightly larger dissociation constant obtained for the heterodimer

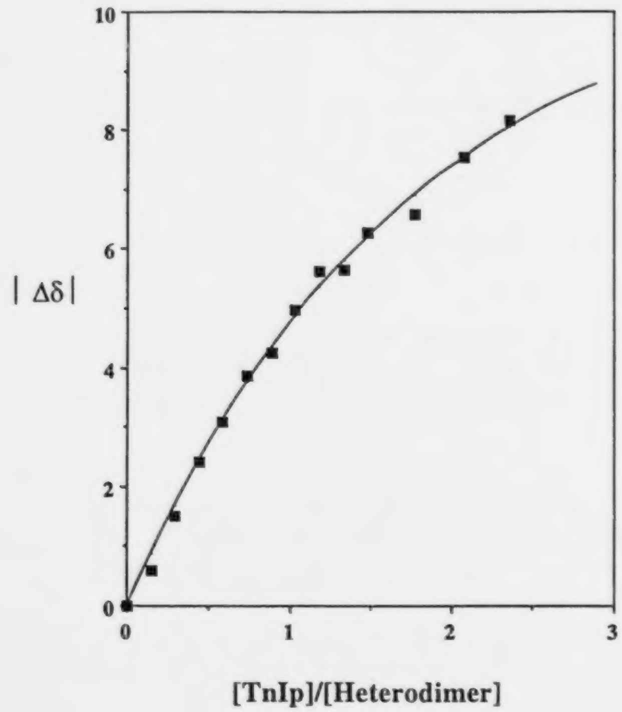

Figure VIII.5: TnIp titration plot for 152.2 μM heterodimer derived from the heterodimer titration data shown in Figure 4. The data are the average absolute values of the chemical shift changes of I113αCH, Y112αCH, D150αCH, I149αCH, I104γCH$_3$, I121γCH$_3$ and I113δCH$_3$ as TnIp is added to the SCIII/SCIV heterodimer. The curve for the binding was calculated using an iterative non-linear least-squares analysis allowing the K_d and the total shift parameters to be simultaneously changed to achieve a best fit giving a value for K_d = 192 ± 37 μM.

indicates that the peptide is bound more tightly to s-TnC perhaps because of additional interactions with the N-terminal domain (see below).

The residues which are perturbed in the C-terminal domain of s-TnC upon binding of TnIp based upon the TnIp titration studies with s-TnC and the SCIII/SCIV heterodimer are all located in a hydrophobic pocket. These residues are surrounded by acidic residues (for color diagram see Figure 2a, Strynadka and James, 1990). In the bound structure of TnIp (Campbell and Sykes, 1991b), the hydrophobic residues of TnIp form a surface which is surrounded by the basic residues of the TnIp. This result certainly suggests that a prime spot for TnIp binding is the C-terminal domain hydrophobic pocket of s-TnC. The F106NH resonance of TnIp is the most shifted amide resonance of TnIp upon binding to s-TnC (Campbell and Sykes, 1991b). This supports the proposed interaction of the hydrophobic surface of the TnIp with the hydrophobic region in C-terminal domain of s-TnC. The fact that the s-TnC residues are perturbed upon TnIp binding demonstrates that the protein must change in some manner to accomodate the TnIp, possibly by opening slightly to more expose the hydrophobic residues. At this point, however, it is unproven whether TnIp actually interacts directly with these residues in the hydrophobic pocket or if TnIp merely binds somewhere else and perturbs these residues. A major influence on chemical shift in this region of s-TnC is the ring current effects of F102, F151, and F154 and it is possible that the alteration of the disposition of one of these aromatic rings could be enough to affect all of the residues discussed above. Detailed NOE experiments should reveal where the site of TnIp binding is if peptide-protein NOE's can be observed. Several reports suggest that the binding of the TnI peptide is to the N-terminal portion of site III (Weeks and Perry, 1978; Grabarek et al., 1981; Leavis et al., 1978; Chong and Hodges, 1981; Wang and Cheung, 1984; Leszyk et al., 1987; Lan et al., 1989; Tao et al., 1986, 1989)). Certainly, this region is highly conserved in s-TnC and CaM and is fairly acidic (Figure VIII.1). Crosslinking studies have indicated that Gly 104 of TnIp interacts with methionine 155 of rabbit skeletal TnC (Ngai et al., 1994). Recently, a model of the interaction between the C-terminal domain of TnC and TnI has been proposed which involves an antiparallel arrangement of TnIp in the hydrophobic pocket of the C-terminal domain of TnC (Ngai et al., 1994). This model is similar to one which was developed based on the binding of the MLCK peptide to calmodulin (Slupsky, C.M., unpublished results).

These results do not preclude additional possible interactions of TnIp with the N-terminal region of s-TnC. A shift of the δCH2 protons of F26/75 (in the N-terminal domain) was seen, but no other assignable shifts were observed. Crosslinking studies suggest that there is an interaction (Kobayashi et al., 1991; Leszyk et al., 1990) between the N-terminal domain of s-TnC and TnIp. Because the N-terminal region of s-TnC contains the regulatory sites, it seems probable that there must be some interaction. Perhaps the C-terminal domain serves to anchor the TnIp portion of TnI to s-TnC and regulation comes by interaction of an extended portion of TnIp or another portion of TnI with the regulatory sites. Extensive NMR studies of CaM complexed to the myosin light chain kinase (MLCK) M13 peptide have shown an interaction of the peptide with both domains of CaM (Ikura et al., 1992). The residues in the C-terminal domain of CaM with which the M13 peptide interacts are analogous to some of the residues observed here to shift upon binding of TnIp (namely F102, I104, I113, and I121 of s-TnC). The M13 peptide also interacts with the N-terminal domain of CaM changing the overall shape of CaM in complex versus CaM alone. This interaction, however, relies on the M13 peptide being entirely helical with a 12 residue span between the first residue which interacts with the C-terminal domain of CaM, and the residue which interacts with the N-terminal domain of CaM. Shortened peptides that do not contain this 12 residue amino acid span only bind to the C-terminal domain of CaM (Kataoka et al., 1991). These shortened peptides could interact with CaM in an analogous manner to the TnIp interaction with TnC.

The results here suggest that the SCIII/SCIV heterodimer is a good model to study the C-terminal domain of s-TnC and its interaction with other muscle proteins. Synthetic peptides representing specific portions of a protein not only aid in our understanding of the folding properties of proteins but allow us to observe the interactions of specific portions of proteins with other proteins. As well, this type of research lays the groundwork for the design of novel proteins with desired activities.

In conclusion, we have compared the residues in the SCIII/SCIV heterodimer and in turkey s-TnC involved in binding the inhibitory TnI peptide and have found the interactions to be similar. We have previously determined the structure of the TnI peptide when bound to calcium-saturated s-TnC (Campbell and Sykes, 1991b), and here we attempt to bring together the structure and the location of binding of the TnI peptide in a model of the interaction of the inhibitory region of TnI with calcium-saturated s-TnC using synthetic peptide models and whole s-TnC. The relief of inhibition of actomyosin ATPase when calcium-saturated s-TnC forms a 1:1 complex with TnI is a key step in the initiation of muscle contraction. An understanding of the interaction between s-TnC and TnI at the molecular level is the key to understanding muscle contraction.

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Chapter IX: *Conclusions*

*M*uscle contraction is triggered by the release of calcium from the sarcoplasmic reticulum after depolarization of the sarcolemma by a nerve impulse. It is the binding of calcium by the thin filament protein troponin C (TnC) which results in the sliding of the thick and thin filaments past one another. For many years, the conformational change that takes place in TnC upon calcium binding to the low affinity calcium-binding sites has been studied. This thesis presents the structure of the calcium-saturated form of TnC solved using a combination of the modern molecular biological techniques of cloning, expression, and isotopic labeling with the modern 3D NMR techniques for assignment and structure elucidation, offering yet another piece with which to solve the puzzle of muscle contraction. This thesis also suggests that modification of cysteine with a small sulfhydryl reagent, and the use of TFE as a denaturant of quaternary structure has little effect on the secondary or tertiary structure of this protein. Finally, it is suggested that the inherent flexibility of the central helix in TnC results in differing orientations of the two domains with respect to one another, allowing TnC to interact with TnI or peptides thereof in different ways than would be suggested by the crystal structure of half-saturated TnC.

Chapters IV and V illustrate two important points. First, in Chapter IV, it was shown that the modification of cysteine 101 of TnC with a sulfhydryl reagent resulted in minimal perturbation of TnC in terms of the secondary or tertiary structure. As well, the modification of TnC resulted in minimal perturbation of the calcium binding properties of the C-terminal domain. These results were important to validate the use of the carboxamidomethyl group in order to prevent oxidation of TnC. In Chapter V, it was shown that TnC dimerizes via the N-terminal domain of one monomer interacting with the N-terminal domain of another monomer. This dimerization was suggested to involve the two N-terminal domain hydrophobic pockets. One other important outcome of

Chapter V was that the solvent 2,2,2-trifluoroethanol (TFE) could be used in small concentrations to overcome the dimerization of TnC. It was shown that the addition of the solvent TFE to a concentration of 15% v/v results in a 10 fold increase in the dimer dissociation constant of calcium-saturated TnC. Further, at the concentrations used in Chapter V, TFE acted to perturb the quaternary structure of TnC without adversely affecting the secondary or tertiary structure as evidenced by minimal changes to its CD spectra and ^{1}H, ^{13}C, and ^{15}N NMR chemical shifts. These results are important since protein aggregation is a problem which is not specific to TnC. Other calcium-binding proteins, such as calcineurin (Anglister et al., 1993; Grzesiek and Bax, 1993; Davies and Riechmann, 1994) or S100 (Gary Shaw, personal communication) also aggregate. It was shown that the aggregation of calcineurin could be alleviated using the detergent CHAPS. One potential problem with using CHAPS, however, is that the molecular weight of this detergent is 615 g/mol versus 103 g/mol for TFE. Therefore, binding of this detergent to a protein could result in a significant decrease in rotational correlation time which would mean broader lines, shorter T_2's, and greater spin diffusion. The binding of TFE to a protein would have a similar effect, but to a lesser extent since it has a molecular weight one-sixth that of CHAPS. TFE also has potential problems associated with its use. TFE has been characterized as a structure-enhancing cosolvent, as it stabilizes a variety of structures in peptides. It must be emphasized, however, that high concentrations of TFE were used to induce structures in peptides (much greater than 15% v/v), and small peptides are generally not as stable as large proteins in terms of structure. 15% v/v TFE has previously been shown to have minimal effect on the structure of lysozyme (Buck et al., 1993), and has now been shown to have a minimal effect on the structure of TnC (Chapter VII).

The three-dimensional structure of the calcium-saturated TnC monomer was solved using NMR techniques, and was shown in Chapters VI and VII. The secondary structure of calcium-saturated TnC was shown to be essentially equivalent to that obtained for the crystal structure of half-saturated TnC, except for residue E41 located in helix B. In the crystal structure (Herzberg and James, 1988; Satyshur et al., 1988, 1994), and in the secondary and tertiary structures of apo NTnC (Gagné et al., 1994, 1995), helix B has a break or kink at residue E41 (in the crystal structure, the ϕ/ψ dihedral angles are -96°/-7°). In the structure of calcium-saturated TnC (Chapters VI and VII) and calcium-saturated NTnC (Gagné et al., 1994, 1995), E41 has helical ϕ/ψ angles. The calcium-saturated structure of calmodulin also has helical ϕ/ψ angles. for the equivalent residue E31 (Babu et al., 1988; Chattopadhyaya et al., 1993; Ikura et al., 1991). A comparison of the chemical shifts for the identical residues in calcium-saturated TnC (in 15% TFE), NTnC (dimer), and calmodulin reveals a very high homology. The structure of the N-terminal domain of calcium-saturated TnC (residues 5 to 83 or 16 to 83) is also very homologous to the NTnC dimer (residues 5 to 83) and calmodulin (residues 6 to 73), as the root-mean-square-deviation for the backbone heavy atoms between the structures are 1.28 Å and 1.25 Å respectively for the residues indicated above. These low RMSD values suggest that the structure of the isolated N-terminal domain of TnC in its dimeric calcium-saturated form and the structure of the N-terminal domain of calcium-saturated calmodulin very closely resemble the N-terminal domain of calcium-saturated TnC. The similarity in structure between the NTnC dimer and the TnC monomer, both structures solved independently, suggest that the structures are correct and further suggest that they represent the true structures presented to TnI during muscle contraction. Since the root-mean-square deviation between the N-terminal domain of TnC and the model for the N-terminal domain of TnC (Herzberg et al., 1986) is 2.25 Å, the model structure appears to be quite different. Indeed, the model structure was modeled after the C-terminal calcium-saturated domain of TnC which is different from the N-terminal calcium-saturated form of TnC. Comparison of the C-terminal domain of TnC with that of the crystal structure (residues 98 to 155) reveals an RMSD of 1.22 Å, whereas comparison of the C-terminal domain of TnC (98 to 155) with the C-terminal domain of calmodulin (residues 85 to 142) reveals an RMSD of 1.67 Å. This suggests that the solution structure of the calcium-saturated C-terminal domain is more similar to the crystal structure than to calmodulin.

Studies of apo calmodulin have revealed that the two domains of this protein are similar to the apo N-terminal domain of TnC (Ad Bax, personal communication). That is at residues E31 and E104 of calmodulin, irregular ϕ, ψ angles which are similar to those found in the apo N-terminal domain of TnC (residue E41), are present. The RMSD between the apo N-terminal domain of TnC and the apo N-terminal domain of calmodulin is approximately 1.3 Å (Ad Bax, personal communication). Thus, the results presented in this thesis suggest that there is a general mechanism for the activation of the two proteins TnC and calmodulin which may form their own family of calcium binding proteins. The binding of calcium to both domains of calmodulin, and to the N-terminal domain of TnC result in a conformational change which involves a reorientation of the helices such that the structures adopt a more open form free to interact with a target protein. The reorientation involves a general movement of the B/C helices (and the F/G helices of the C-terminal domain of calmodulin) away from the A/D helices (and the E/H helices of the C-terminal domain of calmodulin), and an opening of a hydrophobic pocket. The C-terminal domain of TnC is different than that of calmodulin in the absence of calcium because the C-terminal domain of TnC is unstructured. The C-terminal domain of TnC, however, can bind calcium and thus when calcium is removed from the muscle cell during relaxation, the C-terminal sites of TnC can be filled with magnesium, and thus cause TnC to remain firmly anchored to the thin filament. In contrast, the C-terminal domain of calmodulin changes structure significantly, and therefore this could potentially disrupt the anchoring of calmodulin to the thin filament thereby accounting for some of the differences of the two proteins in terms of substitution into the thin filament and measurement of the maximal contractility.

In Chapter VIII, it was proven that the interaction between the inhibitory peptide of TnI with TnC primarily involved the C-terminal domain of TnC rather than the N-terminal domain. It has been shown (Blechner et al., 1992) that binding of TnIp to TnC results in a breakup of the TnC dimer. This suggests that there must be some sort of interaction with the N-terminal domain of TnC. Cross-linking results have suggested that the acidic residues of helix C interact with the basic residues of the inhibitory peptide, and therefore this interaction could be responsible for disrupting the dimer formation. Figure IX.1 illustrates the possible interaction between TnC and the inhibitory portion of TnIp. The model was generated using a docking program which utilizes a monte carlo approach. TnIp was docked onto the C-terminal domain of TnC by orienting TnIp in a similar manner as the MLCK peptide is oriented into calmodulin (Ikura et al., 1992). The model of TnIp binding to the C-terminal domain was similar to the one obtained based upon cross-linking studies (Ngai et al. 1994). The N-terminal domain of the model structure of TnC (Herzberg et al., 1986) was subsequently oriented with the negative C-helix residues over the basic peptide residues, and the complex was further subjected to monte carlo docking followed by attachment of the interdomain linker, alteration of some of the ϕ,ψ angles in the linker region, and energy minimization. The actual structure of TnC bound to TnI may be slightly different than this model, however the model illustrates that a flexible central helix is required for the C-helix of TnC to be able to interact with, or come close to, the basic residues of TnIp.

It is therefore further suggested that TnC and calmodulin form their own group of calcium-binding proteins which have two aspects in common. First, upon calcium binding, the hydrophobic pocket is opened up to produce a large hydrophobic area which causes the proteins to interact with their targets. TnC and calmodulin are different from the calbindins, parvalbumins, and S100's since these proteins do not alter much of their structure upon calcium binding. Calbindin, parvalbumin, and S100 proteins should be grouped together as calcium buffers as they do not, in general, interact with target proteins. Second, both TnC and calmodulin have a flexible linker between the two domains which allows the two domains to adopt any orientation with respect to one another. This feature makes it possible for the two domains to wrap around another protein (such as calmodulin with the myosin light chain kinase molecule (Ikura et al., 1992)), or to remain extended (as suggested by Olah and Trewhella, 1994) but reorient the two domains such that helix C (acidic residues) can interact electrostatically with the inhibitory region of TnI (basic residues).

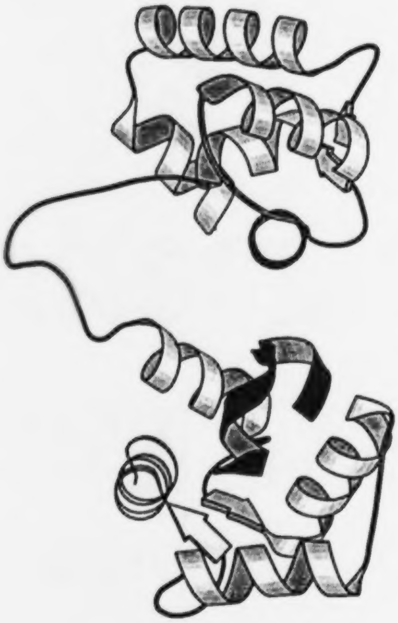

Figure IX.1 A model of the interaction of TnC with TnIp. TnIp is illustrated by the dark ribbon. Helix C is oriented such that it extends across TnIp. The figure was prepared using the program MOLSCRIPT (Kraulis, 1991).

The results presented here bring the understanding of muscle contraction at the molecular level one step up. In order to fully understand the complex nature of muscle contraction, more studies need to be accomplished involving fragments of TnI. The development of 3D and 4D NMR techniques in this laboratory will allow the study of significantly larger fragments of TnI interacting with TnC. Labelling of one component of the complex (such as TnI or TnC) will allow the structure of one to be determined in the presence of the other. Further, since it is clear that the structure of both domains are independent of one another, the domains may be studied by themselves in complex with fragments of TnI to further aid in the understanding of the complex nature of the events which result in muscle contraction.

As more information becomes available, it may occur that calcium binding proteins will be further grouped according to the structural change that happens upon calcium binding. It is possible that the calcium binding proteins of the 'EF'-hand type could be classified as calcium buffers (parvalbumin, calbindin, S100), or calcium-induced activators (TnC, calmodulin).

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Appendix A:

Pulse Sequence Code.

1. ^{15}N-^{1}H HSQC:

```
/* overbdn1.c - heteronuclear Overbodenhausen experiment using REVINEPT

Parameters:
        sspul = 'y': selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence n': normal
            experiment
        fad = 'y': TPPI axial-peak displacement 'n': standard phasecycle
        f1180 = 'y': the first t1 point is sampled at half the t1 dwell time n': the first t1 point is sampled at t1 =
            0
        satflg = 'yn': presaturation during relaxation period (satdly) with xmtr 'nn': no presaturation during
            relaxation period (satdly) 'ny': presaturation during only the null period
        satfrq = presaturation frequency
        satdly = saturation time during the relaxation period
        satpwr = saturation power for all periods of presaturation with xmtr
        hs = 'yn': homospoil pulse (hst) during the d1 relaxation delay
        null = delay associated with the BIRD nulling
        tpwr = power level for 1H transmitter pulses
        pw = 90 degree xmtr pulse length for protons (the observed nucleus)
        pwx2lvl = power level for X decoupler pulses
        pwx2 = 90 degree decoupler pulse length for X
        jxh = one-bond heteronuclear coupling constant to X (in Hz)
        deltaxh = 1/(4*jxh) if jxh != 0.0; otherwise, the entered value is used; the delay used in the REVINEPT
            subsequences
        dm(dm2) = 'nnnnn': no broadband decoupling of X during acquisition 'nnnny': broadband
            heteronuclear decoupling of X during acquisition
        phase = 1,2: hypercomplex experiment with F1 quadrature (complex F1-FT)

modified by bds and dsw on jan. 23, 1992 to correct deltaxh = 0.5*bird
modified by cms on Jan 14, 1993 to include an optional spinlock purge pulse
*/

#include <standard.h>
#include <math.h>

#define MIN_J           0.1         /* Hz  */
#define MIN_DELAY       0.2e-6      /* sec */
#define MIN_NULL        0.0001      /* sec */
#define MAX_SSTRIM      0.1         /* sec */

static int      phs1[4]  = {1,1,3,3},
                phs2[2]  = {0,2},
                phs3[8]  = {0,0,0,0,2,2,2,2},
                phs4[16] = {0,0,0,0,0,0,0,0,2,2,2,2,2,2,2,2},
                phs5[16] = {0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0};
```

```
static double        d2_init = 0.0;

/*---------------------------------------------
|                                             |
|          pulsesequence()/0                  |
|                                             |
+--------------------------------------------*/
pulsesequence()
{
/* VARIABLE DECLARATION */
  char satflg[MAXSTR],
                    sspul[MAXSTR],
                    fad[MAXSTR],
                    f1180[MAXSTR],
                    spinlck[MAXSTR];         /* flag for 1H spinlock pulse */

  int  phase,
                    satmove,
                    t1_counter;

  double ss,
                    sstrim,             /* in ms */
                    t1evol,
                    pwx2lvl,
                    jxh,
                    deltaxh,
                    bird,
                    null,
                    sltime,       /* time for spin lock pulse */
                    slpwr;        /* spin lock power */

/* Load variables */
  satfrq = getval("satfrq");
  satdly = getval("satdly");
  satpwr = getval("satpwr");
  pwx2lvl = getval("pwx2lvl");
  pwx2 = getval("pwx2");
  jxh = getval("jxh");
  deltaxh = getval("deltaxh");
  ss = getval("ss");
  sw1 = getval("sw1");
  sstrim = getval("sstrim");
  null = getval("null");
  sltime = getval("sltime");
  slpwr = getval("slpwr");
  phase = (int) (getval("phase") + 0.5);

  getstr("sspul", sspul);
  getstr("f1180", f1180);
  getstr("fad", fad);
  getstr("satflg", satflg);
  getstr("spinlck", spinlck);

/* Load phase tables */
  settable(t1, 4, phs1);
  settable(t2, 2, phs2);
  settable(t3, 8, phs3);
  settable(t4, 16, phs4);
  settable(t5, 16, phs5);

/* Adjust delays */
  if (jxh > MIN_J)
  {
    bird = 1/(2*jxh);
    deltaxh = 0.5*bird;
  }
  else
  {
    bird = 0.0;
  }

/* Check for 1H frequency change */
  satmove = ( fabs(tof - satfrq) >= 0.1 );

/* Check for correct 'dm/dm2' and 'dpwr/dpwr2' settings */
    if ( (dm2[A] == 'y') || (dm2[B ] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y') )
    {text_error("DM2 must be set to either 'nnnnn' or 'nnnny'\n");
      abort(1);
    }

  if (sstrim > MAX_SSTRIM)
  {text_error("`sstrim` is > maximum value\n");
    abort(1);
  }

/* Determine steady-state mode */
  if (ss < 0)
  {
    ss *= (-1);
    initval(ss, ssval);
    initval(ss, ssctr);
  }

/* Phase incrementation for hypercomplex 2D data */
  if (phase == 2)
    tsadd(t2, 1, 4);

/* FAD phase incrementation */
  if (fad[A] == 'y')
  {
    if (ix == 1)
      d2_init = d2;
    t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
    if (t1_counter % 2)
    {
      tsadd(t2, 2, 4);
      tsadd(t5, 2, 4);              /* receiver phase cycle */
    }
  }

/* BEGIN ACTUAL PULSE SEQUENCE CODE */
  status(A);
    rlpower(tpwr, TODEV);
    rlpower(pwx2lvl, DO2DEV);
    if (sspul[A] == 'y')
    {
      rgpulse(sstrim, zero, rof1, 1.0e-6);
      rgpulse(sstrim, one, rof1, rof2);
      hsdelay(d1);
    }
    else
    {
      hsdelay(d1);
    }
```

```
/* selective saturation period */
    if (satflg[A] == 'y')
    {
      if (satmove)
        offset(satfrq, TODEV);
      rlpower(satpwr, TODEV);
      rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
      if (satmove)
        offset(tof, TODEV);
      rlpower(tpwr, TODEV);
      delay(1.0e-5);
    }

  status(B);
/* Bird pulse and nulling period for both C13 and N15 */
    if (null > MIN_NULL)
    {
      rgpulse(pw, zero, rof1, 0.0);
      delay(bird - rof1 - 1.0e-6 - 2*pwx2 - 0.5*pw);
      rcvroff();
      dec2rgpulse(pwx2, one, rof1, 0.0);
      sim3pulse(2*pw,0.0, 2*pwx2, zero, zero, zero, 1.0e-6, 0.0);
      dec2rgpulse(pwx2, one, 1.0e-6, 0.0);
      rcvron();
      delay(bird - rof1 - 1.0e-6 - 2*pwx2 - 0.5*pw);
      rgpulse(pw, two, rof1, 1.0e-6);
      if (satflg[B] == 'y')
      {
        if (satmove)
          offset(satfrq, TODEV);
        rlpower(satpwr, TODEV);
        rgpulse(null, zero, 1.0e-5, 0.2e-6);
        if (satmove)
          offset(tof, TODEV);
        rlpower(tpwr, TODEV);
        delay(1.0e-5);
      }
      else
      {
        delay(null);
      }
    }

  status(C);
    rcvroff();
    rgpulse(pw, zero, rof1, 0.0);
    dec2phase(zero);
    txphase(zero);
    delay(deltaxh - pwx2);
    sim3pulse(2*pw,0.0, 2*pwx2, zero, zero, zero, 0.0, 0.0);
    dec2phase(t2);
    if(spinlck[0] == 'y')
    {
      rlpower(slpwr,TODEV);
      delay(deltaxh - pwx2 - POWER_DELAY);
      xmtron();             /* add spin lock */
      delay(sltime);
      xmtroff();
      rlpower(tpwr,TODEV);
    }
    else
```

```
   {
     delay(deltaxh - pwx2);
   }
   txphase(t1);
   sim3pulse(pw, 0.0, pwx2, t1, zero, t2, 0.0, 0.0);
   txphase(zero);
   dec2phase(t4);

/* Calculate t1 delay */
    t1evol = d2;
    if (f1180[A] == 'y')
      t1evol += 0.5/sw1;

    if (t1evol > MIN_DELAY)
    {
      t1evol -= 2*pw + (4*pwx2/M_PI);
      if (t1evol < MIN_DELAY)
        t1evol = 0.0;
    }

    delay(t1evol/2);
    rgpulse(2*pw, zero, 0.0, 0.0);
    txphase(t3);
    delay(t1evol/2);

  status(D);
    sim3pulse(pw, 0.0, pwx2, t3, zero, t4, 0.0, 0.0);
    txphase(zero);
    dec2phase(zero);
    delay( deltaxh - pwx2 - (2*pwx2/M_PI) );
    sim3pulse(2*pw, 0.0, 2*pwx2, zero, zero, zero, 0.0, 0.0);
    rlpower(dpwr2,DO2DEV);              /* X decoupling power level */
    delay(rof2);
    rcvron();
    delay(deltaxh - pwx2 - POWER_DELAY - rof2);

  status(E);
    setreceiver(t5);
}
```

2. ^{15}N-T$_2$ experiment:

```
/* N15 T2 measurement - from Barbato et al., Biochemistry 31, 5269-5278, 1992

        Carolyn Slupsky - January, 1993

Parameters:
            spinlck = flag for spin lock purge pulses
            sltime1 = spin lock delay for first spin lock pulse  (ca. 1 ms)
            sltime2 = spin lock delay for second spin lock pulse  (ca. 12 ms)
            slpwr = spin lock power
            tpwr = transmitter power level during 1H pulses
            n15pwr = power level for N15 pulses
            dpwr2 = power level for N15 decoupling during acquisition
            j = coupling constant
            jnh = delay = 1/4J(NH) which should be approx. 2.25 ms.
            tau = approx. 2.75 ms approx 1/4J(NH)
            del = delay time during CPMG sequence should be << 1/2J
            deltap = del - pw (1H)
            relax = parameter which specifies how long the relaxation time is.
```

```
Set the relax parameter in your data set as follows:  if del = 0.45ms, and deltap = 0.44 then if you set relax =
1,2,3 then you end up with T1 relaxation times of 7.46 ms, 14.92 ms, 22.38 ms etc.  Since the T2 relaxation
time consists of 8 N15 180 deg pulses, 14 del delays, 2 deltap delays (consist of del - pw), and 1 proton 180
deg pulse.

two pulses formerly used for States taken out
proper states introduced (tssub(t2, 1 , 4))
identical to version on 500.
*/

#include <standard.h>
#include <math.h>
#define MIN_DELAY   0.2e-6

   static int  phs1[2] = {1,3},
            phs2[4] = {0,0,2,2},
            phs3[1] = {1},
            phs4[32] = {0,0,0,0,0,0,0,0,
                        0,0,0,0,0,0,0,0,
                        2,2,2,2,2,2,2,2,
                        2,2,2,2,2,2,2,2},
            phs5[16] = {1,1,1,1,1,1,1,1,
                        3,3,3,3,3,3,3,3},
            phs6[8] = {0,0,0,0,1,1,1,1},
            phs7[16] = {0,0,2,2,2,2,0,0,
                        2,2,0,0,0,0,2,2};

   static double d2_init = 0.0;

/*-------------------------------------------
|                                           |
|       pulsesequence()                     |
|-------------------------------------------*/

pulsesequence()
{

/* Variable Declaration */

            char        fad[MAXSTR];

            int         phase,
                        relax,
                        t1_counter,
                        count;

            double      ss,
                              slpwr,
                              t1evol,
                              n15pwr,
                              pwn15,
                              jnh,
                              j,
                              del,
                              deltap,
                              sltime1,
                              sltime2;

/* Load Variables */
            n15pwr = getval("n15pwr");
            pwn15 = getval("pwn15");
```

```
          j = getval("j");
          tau = getval("tau");
          del = getval("del");
          ss = getval("ss");
          sw1 = getval("sw1");
          sltime1 = getval("sltime1");
          sltime2 = getval("sltime2");
          slpwr = getval("slpwr");
          phase = (int) (getval("phase") + 0.5);
          relax = (int) (getval("relax")+0.5);

          getstr("fad", fad);

/* Load Phase Tables */
          settable(t1, 2, phs1);
          settable(t2, 4, phs2);
          settable(t3, 1, phs3);
          settable(t4, 32, phs4);
          settable(t5, 16, phs5);
          settable(t6, 8, phs6);
          settable(t7, 16, phs7);

/*Check conditions*/
          if ((dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y'))
          {
                    text_error("DM2 must be set to either 'nnnnn' or 'nnnny'\n");
                    abort(1);
          }

/*Determine steady-state mode */
          if (ss < 0)
          {
                    ss *= (-1);
                    initval(ss, ssval);
                    initval(ss, ssctr);
          }

/*Phase incrementation for hypercomplex 2D data */
          if (phase == 2)
           tssub(t2, 1, 4);

/* Add in FAD */
          if (fad[A] == 'y')
          {
                    if (ix == 1)
                    d2_init = d2;
                    t1_counter = (int) ((d2 - d2_init)*sw1 + 0.5);
                    if (t1_counter % 2)
                    {
                              tsadd(t2, 2, 4);
                              tsadd(t7, 2, 4);
                    }
          }

/* calculate jnh and deltap */
          jnh = 1/(4*j);
          deltap = del - pw;

/* Begin actual pulse sequence */

status(A);
          rlpower(tpwr, TODEV);
```

```
        rlpower(n15pwr, DO2DEV);
        hsdelay(d1);

status(B);
        rcvroff();
        txphase(zero);
        dec2phase(zero);
        rgpulse(pw, zero, 0.0, 0.0);
        delay(jnh);
        sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, zero, 0.0, 0.0);
        rlpower(slpwr, TODEV);
        delay(jnh - POWER_DELAY);
        xmtron();
        delay(sltime1);
        xmtroff();
        rlpower(tpwr, TODEV);
        txphase(t1);
        dec2phase(t2);
        sim3pulse(pw, 0.0, pwn15, t1, zero, t2, 0.0, 0.0);
        txphase(zero);
        dec2phase(zero);

status(C);
        delay(tau);
        sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, zero, 0.0, 0.0);
        delay(tau);
        dec2phase(t2);
        for (count=0; count<relax; count++)    /* insert CPMG sequence */
        {
                delay(del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(2*del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(2*del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(2*del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(deltap);
                rgpulse(2*pw, zero, 0.0, 0.0);
                delay(deltap);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(2*del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(2*del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(2*del);
                dec2rgpulse(2*pwn15, t2, 0.0, 0.0);
                delay(del);
        }
  dec2phase(t1);
  t1evol=d2;
  if(t1evol > MIN_DELAY)
  {
    t1evol -= 2*pw + (4*pwn15/M_PI);
    if (t1evol < MIN_DELAY)
      t1evol = 0.0;
  }
  delay(t1evol/2);
  rgpulse(2*pw, zero, 0.0, 0.0);
  delay(t1evol/2);
  dec2rgpulse(pwn15, t1, 0.0, 0.0);
  dec2phase(zero);
```

```
    rlpower(slpwr, TODEV);
    xmtron();
    delay(sltime2);
    xmtroff();
    rlpower(tpwr, TODEV);
    dec2rgpulse(pwn15, zero, 0.0, 0.0);
    dec2phase(t4);
    delay(tau);
    sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, t4, 0.0, 0.0);
    dec2phase(t5);
    delay(tau);

  status(D);
    sim3pulse(pw, 0.0, pwn15, zero, zero, t5, 0.0, 0.0);
    txphase(t6);
    dec2phase(zero);
    delay(jnh);
    sim3pulse(2*pw, 0.0, 2*pwn15, t6, zero, zero, 0.0, 0.0);
    delay(jnh);
    rcvron();
    rlpower(dpwr2,DO2DEV);

  status(E);
    setreceiver(t7);
}
```

3. ^{15}N-T$_1$ experiment:

```
/* N15 T1 measurement - from Barbato et al., Biochemistry 31, 5269-5278, 1992

Modified from N15 T2 experiment (C. Slupsky, September 16, 1994), changed the phases of the pulses to
match the T1 experiment of Barbato et al., added scrambling pulse as in George Gray sequence (hscT1 found
in Oct. 93 varian psglib), and did states t1 acquisition as per George Gray........(tssub)
                                        C. Slupsky, F. Sonnichsen, and B. Sykes September 16, 1994.
          Modified by kkr and smg (october 22, 1994) to fix pwr during sltime2.
                    Added parameter sl_pw90.

Parameters:
          sltime1 = spin lock delay for first spin lock pulse  (ca. 1 ms)
          sltime2 = spin lock delay for scramble pulses  (ca. 12 ms)
          sl_pw90 = 90 deg pulse at slpwr
          slpwr = spin lock power (for sltime1)
          tpwr = transmitter power level during 1H pulses
          n15pwr = power level for N15 pulses
          dpwr2  = power level for N15 decoupling during acquisition
          j = coupling constant
          jnh = delay = 1/4J(NH) which should be approx. 2.25 ms.
          tau = approx. 2.75 ms approx 1/4J(NH)
          zeta = delay time during relax should be << 1/2J (ca. 7 ms)
          relax = parameter which specifies how long the relaxation time is.

Set the relax parameter in your data set as follows:
if sltime2=12.5 ms and zeta = 7ms then if you set relax = 0,1,2,3,4 then you end up with T1 relaxation times
of 12.5 ms, 26.5 ms, 40.5 ms, 54.5 ms, 68.5 ms since the T1 relaxation time consists of scrambling pulses
during sltime2, and relax*(180 deg. 1H pulse, + two delays (zeta)).

*/

#include <standard.h>
#include <math.h>
```

```
#define MIN_DELAY  0.2e-6

static int          phs1[8] = {0,0,0,0,2,2,2,2},
                    phs2[32] = {0,0,0,0,0,0,0,0,1,1,1,1,1,1,1,1,2,2,2,2,2,2,2,2,3,3,3,3,3,3,3,3},
                    phs3[2] = {1,3},
                    phs4[4] = {0,0,2,2},
                    phs5[32] = {0,0,0,0,0,0,0,0,1,1,1,1,1,1,1,1,2,2,2,2,2,2,2,2,3,3,3,3,3,3,3,3},
                    phs6[8] = {0,2,2,0,2,0,0,2},
                    phs7[8] = {0,0,0,0,1,1,1,1},
                    phs8[8] = {1,1,1,1,0,0,0,0};

static double d2_init = 0.0;

/*-------------------------------------------
|                                           |
|        pulsesequence()                    |
|------------------------------------------*/

pulsesequence()
{

/* Variable Declaration */

  char  fad[MAXSTR];

  int     phase,
          relax,
          t1_counter,
          count;

  double  ss,
                  cycles,
                  slpwr,
                   t1evol,
                  n15pwr,
                  pwn15,
                  sl_pw90,
                  jnh,
                  j,
                  zeta,
                  sltime1,
                   sltime2;

/* Load Variables */
            n15pwr = getval("n15pwr");
            pwn15 = getval("pwn15");
            sl_pw90 = getval("sl_pw90");
            j = getval("j");
            tau = getval("tau");
            zeta = getval("zeta");
            ss = getval("ss");
             sw1 = getval("sw1");
            sltime1 = getval("sltime1");
            sltime2 = getval("sltime2");
            slpwr = getval("slpwr");
            phase = (int) (getval("phase") + 0.5);
            relax = (int) (getval("relax")+0.5);

            getstr("fad", fad);

/* Load Phase Tables */
```

```
  settable(t1, 8, phs1);
  settable(t2, 32, phs2);
  settable(t3, 2, phs3);
  settable(t4, 4, phs4);
  settable(t5, 32, phs5);
  settable(t6, 8, phs6);
  settable(t7, 8, phs7);
  settable(t8, 8, phs8);

  cycles = sltime2/(32*sl_pw90);
  cycles = 2.0*(double)(int)(cycles/2.0);
  sltime2=cycles*(32*sl_pw90);
  initval(cycles,v1);

/*Check conditions*/
  if ((dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y'))
  {
    text_error("dm2 must be set to either 'nnnnn' or 'nnnny'\n");
    abort(1);
  }

/*Determine steady-state mode */
  if (ss < 0)
  {
    ss *= (-1);
    initval(ss, ssval);
    initval(ss, ssctr);
  }

/*Phase incrementation for hypercomplex 2D data */
  if (phase == 2)
    tssub(t1, 1, 4);

/* Add in FAD */
  if (fad[A] == 'y')
  {
    if (ix == 1)
      d2_init = d2;
    t1_counter = (int) ((d2 - d2_init)*sw1 + 0.5);
    if (t1_counter % 2)
    {
      tsadd(t1, 2, 4);
      tsadd(t6, 2, 4);
    }
  }

/* calculate jnh */
          jnh = 1/(4*j);

/* Begin actual pulse sequence */

  status(A);
    rlpower(tpwr, TODEV);
    rlpower(n15pwr, DO2DEV);
    hsdelay(d1);

  status(B);
    rcvroff();
    txphase(zero);
    dec2phase(zero);
    rgpulse(pw, zero, 0.0, 0.0);
    delay(jnh);
  sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, zero, 0.0, 0.0);
  rlpower(slpwr, TODEV);
  delay(jnh - POWER_DELAY);
  xmtron();
  delay(sltime1);
  xmtroff();
  rlpower(tpwr, TODEV);
  txphase(one);
  dec2phase(t1);
  sim3pulse(pw, 0.0, pwn15, one, zero, t1, 0.0, 0.0);
  txphase(zero);
  dec2phase(t2);

status(C);
  delay(tau);
  sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, t2, 0.0, 0.0);
  delay(tau);
  t1evol=d2;
  if(t1evol > MIN_DELAY)
  {
    t1evol -= 2*pw + (4*pwn15/M_PI);
    if (t1evol < MIN_DELAY)
      t1evol = 0.0;
  }
  delay(t1evol/2);
  rgpulse(2*pw, zero, 0.0, 0.0);
  delay(t1evol/2);
          dec2phase(t3);
          dec2rgpulse(pwn15, t3, 0.0, 0.0);
  for (count=0; count<relax; count++)
  {                          /* incorporation of 1H 180deg pulses */
   delay(zeta);         /* which suppress effects of cross correlation */
   rgpulse(2*pw, zero, 0.0, 0.0);    /* between dipolar and CSA  */
   delay(zeta);                   /* relaxation mechanisms */
           }
  dec2phase(t4);
  if (sltime2>0.0)
           {
           rlpower(slpwr, TODEV);
           starthardloop(v1);
                     rgpulse(8*sl_pw90, t7, 0.0, 0.0);
                        rgpulse(8*sl_pw90, t8, 0.0, 0.0);
                        delay(16*sl_pw90);
  endhardloop();
           rlpower(tpwr, TODEV);
           }
  dec2rgpulse(pwn15, t4, 0.0, 0.0);
  dec2phase(zero);
  delay(tau);
  sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, zero, 0.0, 0.0);
  dec2phase(one);
  delay(tau);

status(D);
  sim3pulse(pw, 0.0, pwn15, zero, zero, one, 0.0, 0.0);
  txphase(t2);
  dec2phase(zero);
  delay(jnh);
  sim3pulse(2*pw, 0.0, 2*pwn15, t2, zero, zero, 0.0, 0.0);
  delay(jnh);
  rcvron();
  rlpower(dpwr2,DO2DEV);
```

```
    status(E);
      setreceiver(t6);
}
```

4. 3D HNCA experiment:

```
/*hncanodec.c - modified HNCA experiment by Kay et al.; uses shifted laminar pulses to selectively excite
the Ca spins while on resonance with the CO spins; on-resonance selective decoupling of the CO spins is
employed during tau(cn) and t1; refocusing of the Ca and CO spins is used during t2; this sequence has RF
duality. No proton decoupling is used (as in hnca.c).

Parameters:

        sspul = 'y': selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence
        f1180 = 'n': standard t1 timing
                'y': modified t1 timing for t1(1) = half the dwell time
        f2180 = 'n': standard t2 timing
                'y': modified t2 timing for t2(1) = half the dwell time
                'n': normal experiment
        fad1 = 'y': TPPI axial-peak displacement along t1
               'n': standard phasecycle
        fad2 = 'y': TPPI axial-peak displacement along t2 (3D experiment)
              'n': standard phasecycle
        satmode = 'nnnn': no H1 presaturation
                  'ynnn': H1 presaturation during relaxation delay
                  'nynn': H1 presaturation during the first tau(cn) period
                  'nnyn': H1 presaturation during the t1 period
                  'nnny': H1 presaturation during the second tau(cn) period
        satfrq = frequency of H1 presaturation for all periods
        satdly = saturation time during the relaxation period
        satpwr = saturation power for all periods of presaturation with xmtr
        hs = 'yn': homospoil pulse (hst) during the d1 relaxation delay
        tpwr = power level for H1 transmitter pulses
        pw = 90 degree xmtr pulse length for protons (the observed nucleus)
        cashape = pattern for shifted laminar pulse on the Ca spins
        pwcalvl = power level for Ca decoupler pulses
        pwca = 90 degree decoupler pulse length for Ca at `pwcalvl`
        cafrq = frequency for Ca spins
        pwcolvl = power level for selective C0 decoupling
        pwco = 90 degree pulse length for selective C0 decoupling at `pwcolvl`
        coshape = decoupling pattern for selective CO decoupling
        cores = tip-angle resolution for selective CO decoupling
        cofrq = frequency for CO spins
        c13dev = RF device for C13
        pwn15lvl = power level for N15 decoupler pulses
        pwn15 = 90 degree decoupler pulse length for N15
        n15dev = RF device for N15
        jnh = one-bond heteronuclear coupling constant to NH (in Hz)
        jcn = one-bond heteronuclear coupling constant to CN (in Hz)
        dpwr(N15) = power level for N15 broadband decoupling
        dpwr(C13) = power level for C13 broadband decoupling
        dm(N15) = 'nn': no broadband decoupling of N15 during acquisition
                  'ny': broadband heteronuclear decoupling of N15 during acquisition
        dm(C13) = 'nn': no broadband decoupling of C13 during acquisition
        phase = 1,2: hypercomplex experiment with F1 quadrature (complex F1-FT)
        phase2 = 1,2: hypercomplex experiment with F2 quadrature (complex F2-FT)
                modified from hnca1FSP.c(S.Farmer,Varian) 1/27/92
*/
```

```
#include <standard.h>
#include <math.h>

#define MIN_DELAY        0.2e-6              /* shortest executable delay */
#define MIN_J            0.1                 /* Hz  */
#define MIN_NULL         0.0001              /* sec */
static int     count = 0,
                    phs1[4]  = {0,0,2,2},
                    phs2[2]  = {0,2},
                    phs3[2]  = {0,1},
                    phs4[2]  = {0,2},
                    phs5[2]  = {0,2},
                    phs6[2]  = {0,1},
                    phs7[64] = {0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0,
                                2,0,0,2,0,2,2,0,0,2,2,0,2,0,0,2,
                                2,0,0,2,0,2,2,0,0,2,2,0,2,0,0,2,
                                0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0};

static double       d2_init = 0.0,
                    d3_init = 0.0,
                    t1adj_1 = 0.0,
                    t1adj_2 = 0.0,
                    tmpd2 = 0.0;

extern double       getvalnwarn();


/*---------------------------------
|                                 |
|     pulsesequence()/0           |
|                                 |
+---------------------------------*/
pulsesequence()
{
/* VARIABLE DECLARATION */
  char       satmode[MAXSTR],
                    sspul[MAXSTR],
                    fad1[MAXSTR],
                    f1180[MAXSTR],
                    f2180[MAXSTR],
                    prtinfo[MAXSTR],
                    cashape[MAXSTR],
                    coshape[MAXSTR],
                    *dmc13,
                    *dmn15;
  int        phase,
                    satmove,
                    t1_counter,
                    exp3D,
                    c13dev,
                    n15dev;
  double      ss,
                    sw1,
                    sw2,
                    delayval,
                    t1evol_1,
                    t1evol_2,
                    d2local,
                    cafrq,
                    cofrq,
          pwcalvl,
          pwca,
            pwcolvl,
            pwco,
            cores,
            pwn15lvl,
            pwn15,
            jnh,
            jcn,
            deltanh,
            deltacn,
            satfrq,
            satdly,
            satpwr;

/* LOAD VARIABLES */
  satfrq = getval("satfrq");
  satdly = getval("satdly");
  satpwr = getval("satpwr");
  cofrq = getval("cofrq");
  cafrq = getval("cafrq");
  pwcalvl = getval("pwcalvl");
  pwca = getval("pwca");
  pwcolvl = getval("pwcolvl");
  pwco = getval("pwco");
  cores = getval("cores");
  pwn15lvl = getval("pwn15lvl");
  pwn15 = getval("pwn15");
  jnh = getval("jnh");
  jcn = getval("jcn");
  ss = getval("ss");
  sw1 = getval("sw1");
  phase = (int) (getval("phase") + 0.5);
  c13dev = (int) (getval("c13dev") + 0.5);
  n15dev = (int) (getval("n15dev") + 0.5);

  getstr("cashape", cashape);
  getstr("coshape", coshape);
  getstr("sspul", sspul);
  getstr("fad1", fad1);
  getstr("satmode", satmode);
  getstr("f1180", f1180);
  getstr("prtinfo", prtinfo);

/* INITIALIZE VARIABLES */
  deltanh = ( (jnh > MIN_J) ? 1/(2*jnh) : 0.0 );
  deltacn = ( (jcn > MIN_J) ? 1/(2*jcn) : 0.0 );

/* LOAD PHASE TABLE */
  settable(t1, 4, phs1);
  settable(t2, 2, phs2);
  settable(t3, 2, phs3);
  settable(t4, 2, phs4);
  settable(t5, 2, phs5);
  settable(t6, 2, phs6);
  settable(t7, 64, phs7);

  setdivnfactor(t3, 32);
  setdivnfactor(t4, 4);
  setdivnfactor(t5, 16);
  setdivnfactor(t6, 8);

/* CHECK CONDITIONS */
  satmove = ( fabs(tof - satfrq) >= 0.1 );
  exp3D = ( (int) (getvalnwarn("ni2") + 0.5) > 0 );

  if ( (n15dev != DODEV) && (n15dev != DO2DEV) )
  {
    text_error("invalid RF device for N15\n");
    abort(1);
  }
  else if ( (c13dev != DODEV) && (c13dev != DO2DEV) )
  {
    text_error("invalid RF device for C13\n");
    abort(1);
  }
  else if (c13dev == n15dev)
  {
    text_error("N15 and C13 RF devices must be different\n");
    abort(1);
  }

  if (satmode[C] == 'y')
  {
    text_error("`satmode` must be 'n' during status C\n");
    abort(1);
  }

  dmn15 = ( (n15dev == DODEV) ? dm : dm2 );
  dmc13 = ( (c13dev == DODEV) ? dm : dm2 );

  if ( (strcmp(dmn15, "n") != 0) && (strcmp(dmn15, "nn") != 0) &&
           (strcmp(dmn15, "ny") != 0) )
  {
    text_error("`dm(N15)` must be to 'n', 'nn', or 'ny'\n");
    abort(1);
  }

  if ( (strcmp(dmc13, "n") != 0) && (strcmp(dmc13, "nn") != 0) )
  {
    text_error("`dm(C13)` must be to 'n' or 'nn'\n");
    abort(1);
  }


/* DETERMINE STEADY-STATE MODE */
  if (ss < 0)
  {
    ss *= (-1);
    initval(ss, ssval);
    initval(ss, ssctr);
  }


/* ADD IN STATES-HABERKORN ELEMENT */
  if (phase == 2)
    tsadd(t2, 1, 4);

  if (exp3D)
  {
    int     phase2;

    phase2 = (int) (getval("phase2") + 0.5);
    if (phase2 == 2)
      tssub(t1, 1, 4);
  }
```

```
/* ADD IN FAD */
 if (fad1[A] == 'y')
 {
  if (ix == 1)
   d2_init = d2;

  t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
  if (t1_counter % 2)
  {
    tsadd(t2, 2, 4);          /* first C13 90 phase cycle    */
    tsadd(t7, 2, 4);          /* receiver phase cycle                  */
  }
 }

 if (exp3D)
 {
  char   fad2[MAXSTR];
  int    t2_counter;

  sw2 = getval("sw2");
  getstr("fad2", fad2);
  getstr("f2180", f2180);

  if (fad2[A] == 'y')
  {
   if (ix == 1)
    d3_init = d3;

   t2_counter = (int) ( (d3 - d3_init)*sw2 + 0.5 );
   if (t2_counter % 2)
   {
     tsadd(t1, 2, 4);     /* first N15 90 phase cycle    */
     tsadd(t7, 2, 4);     /* receiver phase cycle        */
   }
  }
 }


/* BEGIN ACTUAL PULSE SEQUENCE CODE */
 status(A);
  rlpower(tpwr, TODEV);             /*  H1 hard-pulse power level */
  rlpower(pwn15lvl, n15dev);        /* N15 hard-pulse power level          */
  rlpower(pwcolvl, c13dev);         /* CO pulse power            */
  offset(cofrq, c13dev);            /* CO frequency                        */
  if (sspul[A] == 'y')
  {
    rgpulse(200*pw, zero, rof1, 0.0);
    rgpulse(200*pw, one, 0.0, rof2);
  }
    hsdelay(d1);
/* selective saturation period */
  if (satmode[A] == 'y')
  {
   if (satmove)
     offset(satfrq, TODEV);
   rlpower(satpwr, TODEV);
   rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
   if (satmove)
     offset(tof, TODEV);
   rlpower(tpwr, TODEV);
```

```
        delay(1.0e-5);
      }
/* Pulse train */
    rcvroff();
    decphase(zero);         /* CO selective decoupling phase */
    decprgon(coshape, pwco, cores);                                          /* start PRG-dec pattern
running */
/* INEPT transfer from H1 to N15 */
    rgpulse(pw, zero, rof1, 5.0e-6);
    txphase(zero);                        /* H1 phase  */
    dec2phase(t1);              /* N15 phase */
    delay(deltanh - 5.0e-6);
    sim3pulse(2*pw,0.0, pwn15, zero,zero, t1, 0.0, 5.0e-6);
    decon();                              /* turn on CO RF    */
    txphase(one);                         /* H1 phase                  */
    dec2phase(t3);              /* N15 phase                */
    delay(deltanh - 5.0e-6);
    rgpulse(pw, one, 0.0, 0.0);
/* HMQC established between N15 and C13 */
    if (satmode[B] == 'y')
    {
      rlpower(satpwr, TODEV);
      txphase(zero);                      /* H1 phase for presaturation */
      if (satmove)
      {
        offset(satfrq, TODEV);
        rgpulse(deltacn - 2*(POWER_DELAY + OFFSET_DELAY) - POWER_DELAY -
WFG_START_DELAY - PRG_STOP_DELAY - deltanh - pw, zero, 0.0, 0.0);
        offset(tof, TODEV);
      }
      else
      {
        rgpulse(deltacn - 3*POWER_DELAY - WFG_START_DELAY - PRG_STOP_DELAY- deltanh -
pw, zero, 0.0, 0.0);
      }

      rlpower(tpwr, TODEV);
    }
    else
    {
      delay(deltacn - deltanh - pw - POWER_DELAY - WFG_START_DELAY- PRG_STOP_DELAY);
      txphase(zero);
    }
/* Calculate t1 evolution times */
    if (ix == 1)
    {
      t1adj_1 = (2*pwca/M_PI) + pw + WFG_START_DELAY + PRG_STOP_DELAY+ POWER_DELAY;
      t1adj_2 = pwn15 + pw;
      t1evol_1 = (8 * sw1) * ( MIN_DELAY + t1adj_1 );
      t1evol_2 = (8 * sw1) * ( MIN_DELAY + t1adj_2 );
      tmpd2 = ( (t1evol_1 > t1evol_2) ? t1evol_1 : t1evol_2 );
      if (prtinfo[0] == 'y')
      {
        (void) printf( "\nMaximum C13 spectral width = %lf\n",
                  ( (f1180[0] == 'y') ? (sw1/tmpd2) : (2*sw1)/tmpd2 ) );
      }
      count = (int) (tmpd2) + 1;
      tmpd2 = count * (0.5/sw1);
      if (prtinfo[0] == 'y')
      {
        char msge[128];
        (void) sprintf(msge, "count = %d\n", count);
      text_error(msge);
    }
  }
  if ( (f1180[0] != 'y') && (count < 3) )
  {
    t1evol_1 = (d2/4) - t1adj_1;
    t1evol_2 = (d2/4) - t1adj_2;
  }
  else
  {
    if ( (count > 2) || ((f1180[0] == 'y') && (count > 1)) )
    {
      if (ix == 1)
        text_error("WARNING:  sw1 is too large for proper t1 timing\n");
    }
    d2local = d2 + tmpd2;
    t1evol_1 = (d2local/4) - t1adj_1;
    t1evol_2 = (d2local/4) - t1adj_2;
  }
  if (t1evol_1 < MIN_DELAY)
    t1evol_1 = 0.0;
  if (t1evol_2 < MIN_DELAY)
    t1evol_2 = 0.0;
/* Start C13 pulsing */
  decoff();                          /* turn off CO RF              */
  rlpower(pwcalvl, c13dev);                  /* Ca pulse power level              */
  decphase(t2);             /* Ca phase                         */
  decprgoff();              /* shutdown C13 WFG                 */
  decshaped_pulse(cashape, pwca, t2, 0.0, 0.0);
  rlpower(pwcolvl, c13dev);                  /* CO decoupling power level         */
  decphase(zero);           /* CO decoupling phase              */
  decprgon(coshape, pwco, cores);                                  /* turn on CO decoupling
       */
  decon();                           /* turn on CO RF               */
  delay(t1evol_1);
  rgpulse(2*pw, zero, 0.0, 0.0);
  delay(t1evol_2);
  dec2rgpulse(2*pwn15, t3, 0.0, 0.0);
  delay(t1evol_2);
  rgpulse(2*pw, zero, 0.0, 0.0);
  delay(t1evol_1);
  decoff();                          /* turn off CO RF              */
  rlpower(pwcalvl, c13dev);          /* Ca pulse power level        */
  decphase(t4);             /* Ca phase                         */
  dec2phase(zero);                   /* N15 phase                          */
  decprgoff();              /* shutdown C13 WFG                 */
  decshaped_pulse(cashape, pwca, t4, 0.0, 0.0);
  rlpower(pwcolvl, c13dev);                  /* CO decoupling pow er level        */
  decphase(zero);           /* CO decoupling phase              */
  decprgon(coshape, pwco, cores);
  decon();                           /* turn on CO RF               */
/* Remove HMQC between N15 and C13 */
  delayval = deltacn - deltanh - POWER_DELAY - WFG_STOP_DELAY-PRG_STOP_DELAY;
  if (exp3D)
    delayval -= 8*pwca + OFFSET_DELAY + POWER_DELAY + 5.0e-6;
  if (satmode[D] == 'y')
  {
    txphase(zero);                   /* H1 phase                           */
    rlpower(satpwr, TODEV);          /* H1 presaturation power level       */
    if (satmove)
    {
      offset(satfrq, TODEV);
```

```
        rgpulse(delayval - 2*POWER_DELAY - 2*OFFSET_DELAY, zero, 0.0, 0.0);
        offset(tof, TODEV);
      }
      else
      {
        rgpulse(delayval - 2*POWER_DELAY, zero, 0.0, 0.0);
      }
      ( (exp3D) ? txphase(zero) : txphase(t5) );
      rlpower(tpwr, TODEV);
    }
    else
    {
      ( (exp3D) ? txphase(zero) : txphase(t5) );
      delay(delayval);
    }
/* Turn off all CO decoupling */
    decoff();                          /* turn off CO RF              */
    decprgoff();                 /* shutdown C13 WFG            */
/* 3D section for N15 t2 evolution */
    if (exp3D)
    {
      double        t2evol;
      t2evol = d3;
      if (f2180[0] == 'y')
        t2evol += (0.5/sw2);
      offset(cafrq, c13dev);           /* Ca center frequency              */
      rlpower(pwcalvl, c13dev);        /* C13 broadband pulse power        */
      delay(5.0e-6);
      delay(t2evol/2);
      decrgpulse(pwca, zero, 0.0, 0.0);
      simpulse(2*pw, 2*pwca, zero, one, 0.0, 0.0);
      decrgpulse(pwca, zero, 0.0, 0.0);
      txphase(t5);                     /* H1 phase         */
      delay(t2evol/2);
      decrgpulse(pwca, zero, 0.0, 0.0);
      decrgpulse(2*pwca, one, 0.0, 0.0);
      decrgpulse(pwca, zero, 0.0, 0.0);
    }
/* INEPT transfer from N15 back to H1 */
    rgpulse(pw, t5, 0.0, 5.0e-6);
    txphase(t6);                       /* H1 phase  */
    delay(deltanh - pw - 5.0e-6);
    sim3pulse(2*pw,0.0, pwn15, t6,zero, zero, 0.0, 0.0);
    rlpower(dpwr, DODEV);              /* N15 or C13 decoupling power */
    rlpower(dpwr2, DO2DEV);                    /* C13 or N15 decoupling power */
    dec2phase(zero);                   /* N15 decoupling phase */
    decphase(zero);              /* C13 final phase */
    delay(rof2);
    rcvron();
    delay(deltanh - 2*POWER_DELAY - PRG_START_DELAY - rof2);
/* Start N15 broadband decoupling */
  status(B);
    setreceiver(t7);
}
```

```
/* hnca_ct_sl.c  -  HNCA 3D experiment by Kay et al.; uses a frequency-shifted pulse on the CO spins; has
RF duality.  This experiment does constant time in the N15 dimension and optional spinlock purge pulses for
suppression of H2O.

Parameters:
        sspul = 'y':  selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence
        f1180 = 'n':  standard t1 timing
```

```
                'y':  modified t1 timing for t1(1) = half the dwell time
        f2180 = 'n':  standard t2 timing
                'y':  modified t2 timing for t2(1) = half the dwell time
                'n':  normal experiment
        fad1 = 'y':  TPPI axial-peak displacement along t1
               'n':  standard phasecycle
        fad2 = 'y':  TPPI axial-peak displacement along t2 (3D experiment)
               'n':  standard phasecycle
        satmode = 'y':  H1 presaturation during relaxtion delay
        satfrq = frequency of 1H presaturation for all periods
        satdly = saturation time during the relaxation period
        satpwr = saturation power for all periods of presaturation with xmtr
        hs = 'yn':  homospoil pulse (hst) during the d1 relaxation delay
        spinlck = flag to check if spin lock purge pulses are to be used
        sltime1 = spin lock time in ms of first spin lock period
        sltime2 = spin lock time in ms of second spin lock period (should be different than sltime1).
        slpwr = spin lock power level.
        tpwr = power level for 1H transmitter pulses
        pw = 90 degree xmtr pulse length for protons (the observed nucleus)
        dof = should be set to Ca frequency
        pwcalvl = power level for Ca decoupler pulses
        pwca = 90 degree decoupler pulse length for Ca at `pwcalvl`
        pwcolvl = power level for C0 decoupler pulses
        pwco = 90 degree decoupler pulse length for C0 at `pwcolvl`
        co180 = C=O shaped pulse
        hard = hard shaped pulse
        pwn15lvl = power level for N15 decoupler pulses
        pwn15 = 90 degree decoupler pulse length for N15
        dpwr2 = power level for N15 broadband decoupling
        inept = inept delay in sequence (NH scalar coupling)
        const = constant-time delay
        dm = 'nnnnn':  no broadband decoupling of N15 during acquisition
             'nnnny':  broadband heteronuclear decoupling of N15 during acquisition
        dm2 = 'n':  no broadband decoupling of C13 during acquisition
        phase = 1,2:  hypercomplex experiment with F1 quadrature (complex F1-FT)
        phase2 = 1,2:  hypercomplex experiment with F2 quadrature (complex F2-FT)
        modified from hncoca_ct.c(S.Gagne)  07/04/92
        modified to include constant-time in F2 (S.Gagne) 07/04/92
        ref.: Grzesiez and Bax, J. Mag. Reson., 96, 432-440 (1992).
        modified to include spin lock purge pulses (C. Slupsky), Feb, 1993
        corrected problems with phasing in Ca dimension (C. Slupksy), Feb, 1994
*/

#include <standard.h>
#include <math.h>
#define MIN_DELAY         0.2e-6                /* shortest executable delay */
static int  phs1[2] = {0,2},
                  phs2[2] = {1,3},
                  phs3[1] = {0},
                  phs4[16] = {0,0,0,0,1,1,1,1,2,2,2,2,3,3,3,3},
                  phs5[32] = {0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,0,
                             2,2,2,2,2,2,2,2,2,2,2,2,2,2,2,2},
                  phs6[32] = {1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,1,
                             3,3,3,3,3,3,3,3,3,3,3,3,3,3,3,3},
                  phs7[4] = {0,0,2,2},
                  phs8[1] = {1},
                  rec[32] = {0,2,2,0,2,0,0,2,0,2,2,0,2,0,0,2,
                            2,0,0,2,0,2,2,0,2,0,0,2,0,2,2,0};

static double        d2_init = 0.0,
        d3_init = 0.0;
```

```
pulsesequence()
{
/* VARIABLE DECLARATION */
  char co180[MAXSTR],
                       hard[MAXSTR],
                       sspul[MAXSTR],
     spinlck[MAXSTR],
                       fad1[MAXSTR],
                       fad2[MAXSTR],
                       f1180[MAXSTR],
                       f2180[MAXSTR];

  int  phase,
                       satmove,
                       t1_counter,
                       t2_counter,
                       c13dev = DODEV,
                       n15dev = DO2DEV;

double ss,
                       t1evol,
                       t2evol_1,
                       t2evol_2,
                       ni2,
                       pwcalvl,
                       pwca,
                       pwcolvl,
                       pwco,
                       pwn15lvl,
                       pwn15,
      sltime1,
      sltime2,
      slpwr,
                       inept,
                       const;

/* Load variables */
   satfrq = getval("satfrq");
   satdly = getval("satdly");
   satpwr = getval("satpwr");
   sltime1 = getval("sltime1");
   sltime2 = getval("sltime2");
   slpwr = getval("slpwr");
   pwcalvl = getval("pwcalvl");
   pwca = getval("pwca");
   pwcolvl = getval("pwcolvl");
   pwco = getval("pwco");
   pwn15lvl = getval("pwn15lvl");
   pwn15 = getval("pwn15");
   inept = getval("inept");
   const = getval("const");
   ss = getval("ss");
   sw1 = getval("sw1");
   sw2 = getval("sw2");
   ni2 = getval("ni2");
   phase = (int) (getval("phase") + 0.5);
   phase2 = (int) (getval("phase2") + 0.5);

   getstr("sspul", sspul);
   getstr("co180", co180);
   getstr("hard", hard);
   getstr("fad1", fad1);
  getstr("fad2", fad2);
  getstr("satmode", satmode);
  getstr("f1180", f1180);
  getstr("f2180", f2180);
  getstr("spinlck", spinlck);

/* Load phase cycles */
  settable(t1, 2, phs1);
  settable(t2, 2, phs2);
  settable(t3, 1, phs3);
  settable(t4, 16, phs4);
  settable(t5, 32, phs5);
  settable(t6, 32, phs6);
  settable(t7, 4, phs7);
  settable(t8, 1, phs8);
  settable(t9, 32, rec);

/* Check conditions */
  satmove = ( fabs(tof - satfrq) >= 0.1 );
  if ( (dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y') )
  {
    text_error("`dm2` must be to 'nnnnn' or 'nnnny' for N15 decoupling\n");
    abort(1);
  }

  if ( (dm[A] == 'y') || (dm[B] == 'y') || (dm[C] == 'y') || (dm[D] == 'y') || (dm[E] == 'y') )
  {
    text_error("`dm` must be to 'n' for C13\n");
    abort(1);
  }

  if ( (const) <= (((ni2/sw2)/2)+0.0002) )
  {  text_error("'const' must be larger then '(ni2/sw2)/2'");
    abort(1);
  }

/* Determine steady-state mode */
  if (ss < 0)
  {
    ss *= (-1);
    initval(ss, ssval);
    initval(ss, ssctr);
  }

/* Add in States-Haberkorn element */
  if (phase2 == 2)              /* N15 t1 element */
    tsadd(t3, 1, 4);
  if (phase == 2)               /* C13 t2 element */
    tsadd(t7, 1, 4);

/* Add in FAD */
  if (fad2[A] == 'y')           /* for N15 */
  {
    if (ix == 1)
      d3_init = d3;

    t2_counter = (int) ( (d3 - d3_init)*sw2 + 0.5 );
    if (t2_counter % 2)
    {
      tsadd(t3, 2, 4);          /* first N15 90-degree pulse   */
      tsadd(t9, 2, 4);          /* receiver phase cycle               */
    }
  }
  if (fad1[A] == 'y')              /* for C13 */
  {
    if (ix == 1)
      d2_init = d2;
    t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
    if (t1_counter % 2)
    {
      tsadd(t7, 2, 4);      /* first C13a 90-degree pulse   */
      tsadd(t9, 2, 4);      /* receiver phase cycle         */
    }
  }

/* BEGIN ACTUAL PULSE SEQUENCE CODE */
  status(A);
    rlpower(tpwr, TODEV);               /*  H1 hard-pulse power level */
    rlpower(pwn15lvl, n15dev);          /*  N15 hard-pulse power level            */
    rlpower(pwcolvl, c13dev);           /*  CO 180 power              */
    if (sspul[A] == 'y')
    {
      rgpulse(200*pw, zero, rof1, 0.0);
      rgpulse(200*pw, one, 0.0, rof2);
    }
    hsdelay(d1);
/* selective saturation period */
    if (satmode[A] == 'y')
    {
      if (satmove)
        offset(satfrq, TODEV);
      rlpower(satpwr, TODEV);
      rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
      if (satmove)
        offset(tof, TODEV);
      rlpower(tpwr, TODEV);
      delay(1.0e-5);
    }
/* Pulse train */
  status(B);
  rcvroff();
  txphase(zero);       /* 1H phase */
    dec2phase(t1);              /* N15 phase */
    decphase(zero);             /* C13 phase */
    rgpulse(pw, zero, 0.0, 0.0);
    delay(inept);
    sim3pulse(2*pw,0.0, 2*pwn15, zero, zero, t1, 0.0, 0.0);
    dec2phase(t3);              /* N15 phase */
    if (spinlck[0] == 'y')
    {
      rlpower(slpwr, TODEV);
      delay(inept-POWER_DELAY);
      xmtron();
      delay(sltime1);
      xmtroff();
      rlpower(tpwr,TODEV);
    }
    else
    {
      delay(inept);
    }
    txphase(t2);                          /* H1 phase  */
    sim3pulse(pw, 0.0, pwn15, t2, zero, t3, 0.0, 0.0);
    txphase(zero);
```

```
   dec2phase(t4);
   t1evol = d3;       /* N15 is governed by ni2 and sw2 */
   if (f2180[0] == 'y')
     t1evol += 0.5/sw2;
   if (t1evol < MIN_DELAY)
     t1evol = 0.0;
   delay(t1evol/2);
   decshaped_pulse(co180, 2*pwco, zero, 0.0, 0.0);
 status(C);
   decphase(t5);
   rlpower(pwcalvl,c13dev);
   delay(const - POWER_DELAY);
   sim3pulse(2*pw, 2*pwca, 2*pwn15, zero, t5, t4, 0.0, 0.0);
   decphase(t7);
   dec2phase(t6);
   delay(const + 2*pwco - (t1evol/2) );
   sim3pulse(0.0, pwca, pwn15, zero, t7, t6, 0.0, 0.0);
   rlpower(pwcolvl, c13dev);
   decphase(zero);
   dec2phase(zero);
   t2evol_1 = d2/2;              /* C13 is governed by ni and sw1 */
   t2evol_2 = d2/2;              /* C13 is governed by ni and sw1 */
   if (f1180[0] == 'y')
{
     t2evol_1 += 0.25/sw1;
     t2evol_2 += 0.25/sw1;
}
   if ( (t2evol_1 > MIN_DELAY) || (t2evol_2 > MIN_DELAY) )
{
     t2evol_1 -= pwca/2 + pwco + POWER_DELAY + WFG_START_DELAY;
     t2evol_2 -= pwca/2 + pwco + POWER_DELAY + WFG_STOP_DELAY;
     if (t2evol_1 < MIN_DELAY)
       t2evol_1 = 0.0;
     if (t2evol_2 < MIN_DELAY)
       t2evol_2 = 0.0;
}
   delay(t2evol_1);

   sim3shaped_pulse(hard, co180, hard, 2*pw, 2*pwco, 2*pwn15, zero, zero, zero, 0.0, 0.0);
   rlpower(pwcalvl, c13dev);
   delay(t2evol_2);
   sim3pulse(0.0, pwca, pwn15, zero, zero, zero, 0.0, 0.0);
   delay(const);
   sim3pulse(0.0, 2*pwca, 2*pwn15, zero, zero, zero, 0.0, 0.0);
   dec2phase(t8);
   delay(const);
 status(D);
   sim3pulse(pw, 0.0, pwn15, zero, zero, t8, 0.0, 0.0);
   dec2phase(zero);
   delay(inept);
   sim3pulse(2*pw, 0.0, 2*pwn15, zero, zero, zero, 0.0, 0.0);
   if (spinlck[0] == 'y')
   {
     rlpower(slpwr, TODEV);
     delay(inept-POWER_DELAY);
     xmtron();
     delay(sltime2);
     xmtroff();
     rlpower(tpwr,TODEV);
   }
   else
   {
      delay(inept);
    }
    rcvron();
    rlpower(dpwr2, DO2DEV);
  status(E);
    setreceiver(t9);
}
```

5. 3D HN(CO)CA experiment:

```
/* hncocanodec.c - HN(CO)CA 3D experiment by Kay et al.; uses a frequency-shifted pulse on the Ca
spins; has RF duality. No proton decoupling is used (as opposed to hncoca.c)

Parameters:
          sspul = 'y': selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence
          f1180 = 'n': standard t1 timing
                  'y': modified t1 timing for t1(1) = half the dwell time
          f2180 = 'n': standard t2 timing
                  'y': modified t2 timing for t2(1) = half the dwell time
                  'n': normal experiment
          fad1 = 'y': TPPI axial-peak displacement along t1
                 'n': standard phasecycle
          fad2 = 'y': TPPI axial-peak displacement along t2 (3D experiment)
                 'n': standard phasecycle
          satmode = 'y': H1 presaturation during relaxtion delay
          satfrq = frequency of 1H presaturation for all periods
          satdly = saturation time during the relaxation period
          satpwr = saturation power for all periods of presaturation with xmtr
          hs = 'yn': homospoil pulse (hst) during the d1 relaxation delay
          tpwr = power level for 1H transmitter pulses
          pw = 90 degree xmtr pulse length for protons (the observed nucleus)
          dof = should be set to C0 frequency
          pwcalvl = power level for Ca decoupler pulses
          pwca = 90 degree decoupler pulse length for Ca at `pwcalvl`
          cashape = frequency-shifted pulse for Ca excitation
          pwcolvl = power level for C0 decoupler pulses
          pwco = 90 degree decoupler pulse length for C0 at `pwcolvl`
          pwco2lvl = power level for shorter CO pulse
          pwco2 = 90 degree decoupler pulse length for C0 at `pwco2lvl`
          pwn15lvl = power level for N15 decoupler pulses
          pwn15 = 90 degree decoupler pulse length for N15
          dpwr2 = power level for N15 broadband decoupling
          tau1 = first delay in sequence (NH scalar coupling)
          tau2 = second delay in sequence
          tau3 = third delay in sequence
          tau4 = fourth delay in sequence
          dm = 'nnnnn': no broadband decoupling of N15 during acquisition
               'nnnny': broadband heteronuclear decoupling of N15 during acquisition
          dm2 = 'n': no broadband decoupling of C13 during acquisition
          phase = 1,2: hypercomplex experiment with F1 quadrature (complex F1-FT)
          phase2 = 1,2: hypercomplex experiment with F2 quadrature (complex F2-FT)
          modified from hncoca1FSP.c(S.Farmer,Varian) 1/27/92
          cms - changed comments dof2 to dof and dpwr to dpwr2 May 11, 1992.
*/

#include <standard.h>
#include <math.h>

#define MIN_DELAY        0.2e-6              /* shortest executable delay */
```

```
static int   phs1[4] = {0,0,2,2},
                     phs2[2] = {0,2},
                     phs3[8] = {0,0,0,0,2,2,2,2},
                    phs5[16] = {0,0,0,0,0,0,0,0,2,2,2,2,2,2,2,2},
                     rec[16] = {0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0};

static double        d2_init = 0.0,
                     d3_init = 0.0;

pulsesequence()
{
/* VARIABLE DECLARATION */
  char     satmode[MAXSTR],
                     sspul[MAXSTR],
                     fad1[MAXSTR],
                     fad2[MAXSTR],
                     f1180[MAXSTR],
                     f2180[MAXSTR],
                     cashape[MAXSTR];
  int      phase,
                     phase2,
                     satmove,
                     t1_counter,
                     t2_counter,
                     c13dev = DODEV,
                     n15dev = DO2DEV;
  double     ss,
                     t1evol,
                     t2evol_1,
                     t2evol_2,
                     sw1,
                     sw2,
           pwcalvl,
           pwca,
           pwcolvl,
           pwco,
           pwco2lvl,
           pwco2,
           pwn15lvl,
           pwn15,
           satfrq,
           satdly,
           satpwr,
                     tau1,
                     tau2,
                     tau3,
                     tau4;

 /* Load variables */
   satfrq = getval("satfrq");
   satdly = getval("satdly");
   satpwr = getval("satpwr");
   pwcalvl = getval("pwcalvl");
   pwca = getval("pwca");
   pwcolvl = getval("pwcolvl");
   pwco = getval("pwco");
   pwco2lvl = getval("pwco2lvl");
   pwco2 = getval("pwco2");
   pwn15lvl = getval("pwn15lvl");
   pwn15 = getval("pwn15");
   tau1 = getval("tau1");
   tau2 = getval("tau2");
  tau3 = getval("tau3");
  tau4 = getval("tau4");
  ss = getval("ss");
  sw1 = getval("sw1");
  sw2 = getval("sw2");
  phase = (int) (getval("phase") + 0.5);
  phase2 = (int) (getval("phase2") + 0.5);

  getstr("cashape", cashape);
  getstr("sspul", sspul);
  getstr("fad1", fad1);
  getstr("fad2", fad2);
  getstr("satmode", satmode);
  getstr("f1180", f1180);
  getstr("f2180", f2180);

/* Load phase cycles */
  settable(t1, 4, phs1);
  settable(t2, 2, phs2);
  settable(t3, 8, phs3);
  settable(t4, 16, rec);
  settable(t5, 16, phs5);

/* Check conditions */
  satmove = ( fabs(tof - satfrq) >= 0.1 );

  if ( (dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y')
                        || (dm2[D] == 'y') )
  {
    text_error("`dm2` must be to 'nnnnn' or 'nnnny' for N15 decoupling\n");
    abort(1);
  }

  if ( (dm[A] == 'y') || (dm[B] == 'y') || (dm[C] == 'y') || (dm[D] == 'y') || (dm[E] == 'y') )
  {
    text_error("`dm` must be to 'n' for C13\n");
    abort(1);
  }


/* Determine steady-state mode */
  if (ss < 0)
  {
    ss *= (-1);
    initval(ss, ssval);
    initval(ss, ssctr);
  }

/* Add in States-Haberkorn element */
  if (phase2 == 2)              /* N15 t1 element */
    tsadd(t2, 1, 4);
  if (phase == 2)               /* C13 t2 element */
    tsadd(t3, 1, 4);

/* Add in FAD */
  if (fad2[A] == 'y')           /* for N15 */
  {
    if (ix == 1)
      d3_init = d3;
    t2_counter = (int) ( (d3 - d3_init)*sw2 + 0.5 );
    if (t2_counter % 2)
    {
      tsadd(t2, 2, 4);              /* first N15 90-degree pulse  */
      tsadd(t4, 2, 4);              /* receiver phase cycle               */
    }
  }
  if (fad1[A] == 'y')               /* for C13 */
  {
    if (ix == 1)
     d2_init = d2;
    t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
    if (t1_counter % 2)
    {
      tsadd(t3, 2, 4);      /* first C13a 90-degree pulse  */
      tsadd(t4, 2, 4);      /* receiver phase cycle        */
    }
  }

/* BEGIN ACTUAL PULSE SEQUENCE CODE */
  status(A);
    rlpower(tpwr, TODEV);                   /*  H1 hard-pulse power level */
    rlpower(pwn15lvl, n15dev);              /* N15 hard-pulse power level         */
    rlpower(pwcolvl, c13dev);               /* CO hard-pulse power                */
    if (sspul[A] == 'y')
    {
      rgpulse(200*pw, zero, rof1, 0.0);
      rgpulse(200*pw, one, 0.0, rof2);
    }
      hsdelay(d1);
/* selective saturation period */
    if (satmode[A] == 'y')
    {
      if (satmove)
        offset(satfrq, TODEV);
      rlpower(satpwr, TODEV);
      rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
      if (satmove)
        offset(tof, TODEV);
      rlpower(tpwr, TODEV);
      delay(1.0e-5);
    }
/* Pulse train */
  status(B);
    rcvroff();
    rgpulse(pw, zero, rof1, 0.0);
    dec2phase(t1);              /* N15 phase */
    decphase(zero);             /* C13 phase */
    delay(tau1);
    sim3pulse(2*pw,0.0, 2*pwn15, zero,zero, t1, 0.0, 0.0);
    txphase(t1);                            /* H1 phase  */
    dec2phase(t2);              /* N15 phase */
    delay(tau1);
    getelem(t1, ct, v14);
    add(one, v14, v14);
    sim3pulse(pw,0.0, pwn15, v14,zero, t2, 0.0, 0.0);
    txphase(zero);
    dec2phase(zero);
    delay(tau2);
    t1evol = d3;       /* N15 is governed by ni2 and sw2 */
    if (f2180[0] == 'y')
      t1evol += 0.5/sw2;
    if (t1evol > MIN_DELAY)
    {
      t1evol -= 2*pw;
      if (t1evol < MIN_DELAY)
        t1evol = 0.0;
    }
    delay(t1evol/2);
    rgpulse(2*pw, zero, 0.0, 0.0);
    delay(t1evol/2);
  status(C);
    delay(tau3);
    sim3pulse(0.0, 2*pwco,2*pwn15, zero, zero,zero,0.0,0.0);
    delay(tau2 + tau3);
    sim3pulse(0.0,pwco,pwn15, zero, zero,zero, 0.0, 0.0);
    delay(tau4/2);
    rlpower(pwcalvl, c13dev);
    decphase(t3);
    delay( (tau4/2) - POWER_DELAY - WFG_START_DELAY );
    decshaped_pulse(cashape, pwca, t3, 0.0, 0.0);
    t2evol_1 = t2evol_2 = d2/2;             /* C13 is governed by ni and sw */
    if (f1180[0] == 'y')
    {
      t2evol_1 += 0.25/sw1;
      t2evol_2 += 0.25/sw1;
    }
    if ( (t2evol_1 > MIN_DELAY) || (t2evol_2 > MIN_DELAY) )
    {
      t2evol_1 -= (2*pwca/M_PI) + pwco2 + POWER_DELAY +WFG_STOP_DELAY;
      t2evol_2 -= (2*pwca/M_PI) + pwco2 + POWER_DELAY +WFG_START_DELAY;
      if (t2evol_1 < MIN_DELAY)
        t2evol_1 = 0.0;
      if (t2evol_2 < MIN_DELAY)
        t2evol_2 = 0.0;
    }
    decphase(zero);
    rlpower(pwco2lvl, c13dev);
    delay(t2evol_1);
    simpulse(2*pw, 2*pwco2, zero, zero, 0.0, 0.0);
    rlpower(pwcalvl, c13dev);
    delay(t2evol_2);
    decshaped_pulse(cashape, pwca, zero, 0.0, 0.0);
    delay( (tau4/2) - WFG_STOP_DELAY );
    rlpower(pwcolvl, c13dev);
    delay( (tau4/2) - POWER_DELAY );
  status(D);
    sim3pulse(0.0,pwco,pwn15,zero,zero, t5, 0.0, 0.0);
    delay(tau3 + tau2);
    sim3pulse(0.0,2*pwco,2*pwn15,zero, zero, zero, 0.0, 0.0);
    delay(tau3);
    rgpulse(2*pw, zero, 0.0, 0.0);
    delay(tau2);
    sim3pulse(pw,0.0, pwn15, zero,zero, zero, 0.0, 0.0);
    delay(tau1);
    sim3pulse(2*pw,0.0, 2*pwn15, zero, zero,zero, 0.0, 0.0);
    rlpower( ((n15dev == DODEV) ? dpwr : dpwr2), n15dev);
    rlpower( ((c13dev == DODEV) ? dpwr : dpwr2), c13dev);
    delay(rof2);
    rcvron();
    delay(tau1 - rof2 - 2*POWER_DELAY);
  status(E);
    setreceiver(t4);
}
```

```
/* hncoca_sl.c  -  HN(CO)CA 3D experiment by Kay et al.; uses a frequency-shifted pulse on the Ca spins;
has RF duality.  No proton decoupling is used(as opposed to hncoca.c).  Constant-time experiment in f2
```

```
Parameters:
        sspul = 'y':  selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence
        f1180 = 'n':  standard t1 timing
                'y':  modified t1 timing for t1(1) = half the dwell time
        f2180 = 'n':  standard t2 timing
                'y':  modified t2 timing for t2(1) = half the dwell time
                'n':  normal experiment
        fad1 = 'y':  TPPI axial-peak displacement along t1
                'n':  standard phasecycle
        fad2 = 'y':  TPPI axial-peak displacement along t2 (3D experiment)
                'n':  standard phasecycle
        satmode = 'y':  H1 presaturation during relaxtion delay
        satfrq = frequency of 1H presaturation for all periods
        satdly = saturation time during the relaxation period
        satpwr = saturation power for all periods of presaturation with xmtr
        hs = 'yn':  homospoil pulse (hst) during the d1 relaxation delay
        tpwr = power level for 1H transmitter pulses
        pw = 90 degree xmtr pulse length for protons (the observed nucleus)
        dof = should be set to C0 frequency
        pwcalvl = power level for Ca decoupler pulses
        pwca = 90 degree decoupler pulse length for Ca at `pwcalvl`
        cashape = frequency-shifted pulse for Ca excitation
        pwca2lvl = power level for Ca 180 decoupler pulses
        pwca2 = 180 degree decoupler pulse length for Ca at `pwcalvl`
        cashape2 = frequency-shifted pulse for Ca 180 excitation
        pwcolvl = power level for C0 decoupler pulses
        pwco = 90 degree decoupler pulse length for C0 at `pwcolvl`
        pwco2lvl = power level for shorter CO pulse
        pwco2 = 90 degree decoupler pulse length for C0 at `pwco2lvl`
        pwn15lvl = power level for N15 decoupler pulses
        pwn15 = 90 degree decoupler pulse length for N15
        dpwr2 = power level for N15 broadband decoupling
        inept = inept delay in sequence (NH scalar coupling; ~2.4 ms)
        tau1 = first delay in sequence (~8.3 ms)
        tau2 = second delay in sequence (~3.0 ms)
        tau3 = third delay in sequence (~5.5 ms
        dm2 = 'nnnnn':  no broadband decoupling of N15 during acquisition
              'nnnny':  broadband heteronuclear decoupling of N15 during acquisition
        dm = 'n':  no broadband decoupling of C13 during acquisition
        phase = 1,2:  hypercomplex experiment with F1 quadrature (complex F1-FT)
        phase2 = 1,2:  hypercomplex experiment with F2 quadrature (complex F2-FT)
           modified from hncoca1FSP.c(S.Farmer,Varian)  1/27/92
           modified from hncocaxx.c to include constant-time in F2 (S.Gagne) 07/01/92
        ref.: Grzesiez and Bax, J. Magn. Reson., 96, 432-440 (1992).
        NOTE :  SHAPED PULSE : carrier on C=O and shaped pulse on Ca
        ........modified from hncoca_ct.c to include optional spin lock purge pulses for H2O suppression (C.
        Slupsky) Feb 11, 1993
*/

#include <standard.h>
#include <math.h>

#define MIN_DELAY       0.2e-6          /* shortest executable delay */

static int   phs1[4] = {0,0,2,2},
                     phs2[2] = {0,2},
                     phs3[8] = {0,0,0,0,2,2,2,2},
                     phs5[16] = {0,0,0,0,0,0,0,0,2,2,2,2,2,2,2,2},
                     rec[16] = {0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0};
static double        d2_init = 0.0,
                     d3_init = 0.0;
```

```
pulsesequence()
{
/* VARIABLE DECLARATION */
 char        sspul[MAXSTR],
                 fad1[MAXSTR],
                 fad2[MAXSTR],
                 f1180[MAXSTR],
                 f2180[MAXSTR],
                 spinlck[MAXSTR],
                 cashape[MAXSTR],
                 cashape2[MAXSTR];
 int         phase,
                 satmove,
                 t1_counter,
                 t2_counter,
                 c13dev = DODEV,
                 n15dev = DO2DEV;
 double       ss,
                 t1evol,
                 t2evol_1,
                 t2evol_2,
                 ni2,
   pwcalvl,
   pwca,
   pwca2lvl,
   pwca2,
   pwcolvl,
   pwco,
   pwco2lvl,
   pwco2,
   pwn15lvl,
   pwn15,
          sltime1,
          sltime2,
          slpwr,
                 inept,
                 tau2,
                 tau1,
                 tau3;

/* Load variables */
  satfrq = getval("satfrq");
  satdly = getval("satdly");
  satpwr = getval("satpwr");
  pwcalvl = getval("pwcalvl");
  pwca = getval("pwca");
  pwca2lvl = getval("pwca2lvl");
  pwca2 = getval("pwca2");
  pwcolvl = getval("pwcolvl");
  pwco = getval("pwco");
  pwco2lvl = getval("pwco2lvl");
  pwco2 = getval("pwco2");
  pwn15lvl = getval("pwn15lvl");
  pwn15 = getval("pwn15");
  inept = getval("inept");
  tau2 = getval("tau2");
  tau1 = getval("tau1");
  tau3 = getval("tau3");
  ss = getval("ss");
  sw1 = getval("sw1");
  sw2 = getval("sw2");
  ni2 = getval("ni2");
  sltime1 = getval("sltime1");
  sltime2 = getval("sltime2");
  slpwr = getval("slpwr");
  phase = (int) (getval("phase") + 0.5);
  phase2 = (int) (getval("phase2") + 0.5);

  getstr("cashape", cashape);
  getstr("cashape2", cashape2);
  getstr("sspul", sspul);
  getstr("fad1", fad1);
  getstr("fad2", fad2);
  getstr("satmode", satmode);
  getstr("f1180", f1180);
  getstr("f2180", f2180);
  getstr("spinlck",spinlck);

/* Load phase cycles */
  settable(t1, 4, phs1);
  settable(t2, 2, phs2);
  settable(t3, 8, phs3);
  settable(t4, 16, rec);
  settable(t5, 16, phs5);

/* Check conditions */
  satmove = ( fabs(tof - satfrq) >= 0.1 );
  if ( (dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y') )
  {
    text_error("`dm2' must be to 'nnnnn' or 'nnnny' for N15 decoupling\n");
    abort(1);
  }
  if ( (dm[A] == 'y') || (dm[B] == 'y') || (dm[C] == 'y') || (dm[D] == 'y') || (dm[E] == 'y') )
  {
    text_error("`dm' must be to 'n' for C13\n");
    abort(1);
  }
  if ( (tau2+tau1) <= ((ni2/sw2)/2) )
  {  text_error("`tau2+tau1' must be larger then '(ni2/sw2)/2'");
    abort(1);
  }

/* Determine steady-state mode */
  if (ss < 0)
  {
    ss *= (-1);
    initval(ss, ssval);
    initval(ss, ssctr);
  }

/* Add in States-Haberkorn element */
  if (phase2 == 2)             /* N15 t1 element */
    tsadd(t2, 1, 4);
  if (phase == 2)              /* C13 t2 element */
    tsadd(t3, 1, 4);

/* Add in FAD */
  if (fad2[A] == 'y')          /* for N15 */
  {
    if (ix == 1)
      d3_init = d3;
    t2_counter = (int) ( (d3 - d3_init)*sw2 + 0.5 );
    if (t2_counter % 2)
```

```
     {
       tsadd(t2, 2, 4);            /* first N15 90-degree pulse  */
       tsadd(t4, 2, 4);            /* receiver phase cycle                */
     }
   }
   if (fad1[A] == 'y')             /* for C13 */
   {
     if (ix == 1)
      d2_init = d2;
   t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
   if (t1_counter % 2)
     {
      tsadd(t3, 2, 4);      /* first C13a 90-degree pulse  */
      tsadd(t4, 2, 4);      /* receiver phase cycle        */
     }
   }

/* BEGIN ACTUAL PULSE SEQUENCE CODE */
  status(A);
    rlpower(tpwr, TODEV);               /*  H1 hard-pulse power level */
    rlpower(pwn15lvl, n15dev);          /*  N15 hard-pulse power level             */
    rlpower(pwca2lvl, c13dev);          /*  Ca 180 power              */
    if (sspul[A] == 'y')
    {
      rgpulse(200*pw, zero, rof1, 0.0);
      rgpulse(200*pw, one, 0.0, rof2);
    }
      hsdelay(d1);
/* selective saturation period */
    if (satmode[A] == 'y')
    {
      if (satmove)
        offset(satfrq, TODEV );
      rlpower(satpwr, TODEV);
      rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
      if (satmove)
        offset(tof, TODEV);
      rlpower(tpwr, TODEV);
      delay(1.0e-5);
    }

/* Pulse train */
  status(B);
   rcvroff();
   rgpulse(pw, zero, rof1, 0.0);
   dec2phase(t1);              /* N15 phase */
   decphase(zero);             /* C13 phase */
   delay(inept);
   sim3pulse(2*pw,0.0, 2*pwn15, zero,zero, t1, 0.0, 0.0);
   txphase(t1);                           /* H1 phase  */
   dec2phase(t2);              /* N15 phase */
             if (spinlck[0] == 'y')
             {
               rlpower(slpwr,TODEV);
               txphase(zero);
               delay(inept-POWER_DELAY);
                xmtron();
               delay(sltime1);
               xmtroff();
               rlpower(tpwr,TODEV);
             }
             else
  delay(inept);
  getelem(t1, ct, v14);
  add(one, v14, v14);
  sim3pulse(pw,0.0, pwn15, v14,zero, t2, 0.0, 0.0);
  txphase(zero);
  dec2phase(zero);
  t1evol = d3;      /* N15 is governed by ni2 and sw2 */
  if (f2180[0] == 'y')
    t1evol += 0.5/sw2;
  if (t1evol > MIN_DELAY)
  {
    t1evol -= pwn15 + pwca2 + 2*WFG_START_DELAY;
    if (t1evol < MIN_DELAY)
      t1evol = 0.0;
  }
  delay(t1evol/2);
  decshaped_pulse(cashape2,pwca2,zero,0.0,0.0);
  delay(tau1 - pwca2/2 - WFG_STOP_DELAY - pw);
  rgpulse(2*pw, zero, 0.0, 0.0);
status(C);
  delay(tau2/2 - pw);
  rlpower(pwcolvl,c13dev);
  delay( (tau2/2) - POWER_DELAY - pwco );
  sim3pulse(0.0, 2*pwco,2*pwn15, zero, zero,zero,0.0,0.0);
  delay(tau2 + tau1 - pwco - pwco/2 - (t1evol/2) - pwn15/2 - pwca2/2 -WFG_START_DELAY);
  sim3pulse(0.0,pwco,pwn15, zero, zero,zero, 0.0, 0.0);
  delay(tau3/2 - pwco/2);
  rlpower(pwcalvl, c13dev);
  decphase(t3);
  delay( (tau3/2) - POWER_DELAY - WFG_START_DELAY - pwca/2);
  decshaped_pulse(cashape, pwca, t3, 0.0, 0.0);
  t2evol_1 = t2evol_2 = d2/2;         /* C13 is governed by ni and sw */
  if (f1180[0] == 'y')
  {
    t2evol_1 += 0.25/sw1;
    t2evol_2 += 0.25/sw1;
  }
  if ( (t2evol_1 > MIN_DELAY) || (t2evol_2 > MIN_DELAY) )
  {
    t2evol_1 -= pwca/2 + pwco2 + POWER_DELAY + WFG_STOP_DELAY;
    t2evol_2 -= pwca/2 + pwco2 + POWER_DELAY + WFG_START_DELAY;
    if (t2evol_1 < MIN_DELAY)
      t2evol_1 = 0.0;
    if (t2evol_2 < MIN_DELAY)
      t2evol_2 = 0.0;
  }
  decphase(zero);
  rlpower(pwco2lvl, c13dev);
  delay(t2evol_1);
  simpulse(2*pw, 2*pwco2, zero, zero, 0.0, 0.0);
  rlpower(pwcalvl, c13dev);
  delay(t2evol_2);
  decshaped_pulse(cashape, pwca, zero, 0.0, 0.0);
  delay( (tau3/2) - WFG_STOP_DELAY - pwca/2 );
  rlpower(pwcolvl, c13dev);
  delay( (tau3/2) - POWER_DELAY - pwco/2 );

status(D);
  sim3pulse(0.0,pwco,pwn15,zero,zero, t5, 0.0, 0.0);
  delay(tau1 + tau2 - pwco/2 - pwco );
  sim3pulse(0.0,2*pwco,2*pwn15,zero, zero, zero, 0.0, 0.0);
  delay(tau1 - pwco - pw);
```

```
    rgpulse(2*pw, zero, 0.0, 0.0);
    delay(tau2 - pw - pwn15/2);
    sim3pulse(pw,0.0, pwn15, zero,zero, zero, 0.0, 0.0);
    delay(inept);
    sim3pulse(2*pw,0.0, 2*pwn15, zero, zero,zero, 0.0, 0.0);
    rlpower( ((n15dev == DODEV) ? dpwr : dpwr2), n15dev);
    rlpower( ((c13dev == DODEV) ? dpwr : dpwr2), c13dev);
            if (spinlck[0] == 'y')
            {
              rlpower(slpwr,TODEV);
              txphase(zero);
              delay(inept-3*POWER_DELAY);
               xmtron();
              delay(sltime2);
              xmtroff();
              rlpower(tpwr,TODEV);
            }
            else
    delay(inept-2*POWER_DELAY);
    delay(rof2);
    rcvron();

  status(E);
    setreceiver(t4);
}
```

6. 3D HNCO experiment:

```
/* hnconodec.c  -   modified HNCO experiment by Kay et al.; uses shifted laminar pulses for selective
inversion of the Ca spins during t1; uses a broadband inversion pulse to refocus the Ca-N15 and CO-N15
scalar interactions during t2; the C13 and N15 RF channels are user- selectable. No proton decoupling is
used (as opposed to hnco.c)

Parameters:
        sspul = 'y': selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence
        f1180 = 'n': standard t1 timing
                'y': modified t1 timing for t1(1) = half the dwell time
        f2180 = 'n': standard t2 timing
                'y': modified t2 timing for t2(1) = half the dwell time
                'n': normal experiment
        fad1 = 'y': TPPI axial-peak displacement along t1
               'n': standard phasecycle
        fad2 = 'y': TPPI axial-peak displacement along t2 (3D experiment)
               'n': standard phasecycle
        satmode = 'nnnn': no H1 presaturation
                  'ynnn': H1 presaturation during relaxation delay
                  'nynn': H1 presaturation during the first tau(cn) period
                  'nnyn': H1 presaturation during the t1 period
                  'nnny': H1 presaturation during the second tau(cn) period
        satfrq = frequency of H1 presaturation for all periods
        satdly = saturation time during the relaxation period
        satpwr = saturation power for all periods of presaturation with xmtr
        hs = 'yn': homospoil pulse (hst) during the d1 relaxation delay
        tpwr = power level for H1 transmitter pulses
        pw = 90 degree xmtr pulse length for protons (the observed nucleus)
        cafrq = frequency for Ca spins (not explicitly used in the sequence)
        cofrq = frequency for CO spins
        pwcalvl = power level for Ca decoupler pulses
        pwca = 90 degree decoupler pulse length for Ca at `pwcalvl`
        cashape = Ca pulse pattern for generating shifted laminar pulse
```

```
            pwcolvl = power level for C0 decoupler pulses
            pwco = 90 degree decoupler pulse length for C0 at `pwcolvl`
            pwcoalvl = power level for broadband C13 pulse
            pwcoa = 90 degree decoupler pulse length for C13 at `pwcoalvl`
            c13dev = RF device for C13
            pwn15lvl = power level for N15 decoupler pulses
            pwn15 = 90 degree decoupler pulse length for N15
            n15dev = RF device for N15
            jnh = one-bond heteronuclear coupling constant to NH (in Hz)
            jcn = one-bond heteronuclear coupling constant to CN (in Hz)
            dpwr(N15) = power level for N15 broadband decoupling
            dpwr(C13) = power level for C13 broadband decoupling
            dm(N15) = 'nn':  no broadband decoupling of N15 during acquisition
                      'ny':  broadband heteronuclear decoupling of N15 during acquisition
            dm(C13) = 'nn':  no broadband decoupling of C13 during acquisition
            phase = 1,2:  hypercomplex experiment with F1 quadrature (complex F1-FT)
            phase2 = 1,2:  hypercomplex experiment with F2 quadrature (complex F2-FT)
            modified from hnco1FSP.c(S.Farmer,Varian)  1/27/92
*/

#include <standard.h>
#include <math.h>

#define MIN_DELAY       0.2e-6          /* shortest executable delay */
#define MIN_J           0.1             /* Hz  */
#define MIN_NULL        0.0001          /* sec */

static int  count = 0,
                phs1[4]  = {0,0,2,2},
                phs2[2]  = {0,2},
                phs3[2]  = {0,1},
                phs4[2]  = {0,2},
                phs5[2]  = {0,2},
                phs6[2]  = {0,1},
                phs7[64] = {0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0,
                            2,0,0,2,0,2,2,0,0,2,2,0,2,0,0,2,
                            2,0,0,2,0,2,2,0,0,2,2,0,2,0,0,2,
                            0,2,2,0,2,0,0,2,2,0,0,2,0,2,2,0};

static double   d2_init = 0.0,
                d3_init = 0.0,
                t1adj_1 = 0.0,
                t1adj_2 = 0.0,
                t1adj_3 = 0.0,
                t1adj_4 = 0.0,
                tmpd2 = 0.0;

extern double   getvalnwarn();

/*------------------------------
|                              |
|   pulsesequence()/0      |
|                              |
+-----------------------------*/
pulsesequence()
{
/* VARIABLE DECLARATION */
  char      satmode[MAXSTR],
                sspul[MAXSTR],
                cashape[MAXSTR],
                fad1[MAXSTR],
                f1180[MAXSTR],
                        f2180[MAXSTR],
                        prtinfo[MAXSTR],
                        *dmc13,
                        *dmn15;
    int         phase,
                        satmove,
                        c13pwrmove,
                        t1_counter,
                        exp3D,
                        c13dev,
                        n15dev;
    double       ss,
                        sw1,
                        sw2,
                        delayval,
                        t1evol_1,
                        t1evol_2,
                        t1evol_3,
                        t1evol_4,
                        d2local,
                        cofrq,
            pwcoalvl,
            pwcoa,
            pwcalvl,
            pwca,
            pwcolvl,
            pwco,
            pwn15lvl,
            pwn15,
            jnh,
            jcn,
            deltanh,
            deltacn,
            satfrq,
            satdly,
            satpwr;

/* LOAD VARIABLES */
  satfrq = getval("satfrq");
  satdly = getval("satdly");
  satpwr = getval("satpwr");
  cofrq = getval("cofrq");
  pwcoalvl = getval("pwcoalvl");
  pwcoa = getval("pwcoa");
  pwcalvl = getval("pwcalvl");
  pwca = getval("pwca");
  pwcolvl = getval("pwcolvl");
  pwco = getval("pwco");
  pwn15lvl = getval("pwn15lvl");
  pwn15 = getval("pwn15");
  jnh = getval("jnh");
  jcn = getval("jcn");
  ss = getval("ss");
  sw1 = getval("sw1");
  phase = (int) (getval("phase") + 0.5);
  c13dev = (int) (getval("c13dev") + 0.5);
  n15dev = (int) (getval("n15dev") + 0.5);

  getstr("sspul", sspul);
  getstr("fad1", fad1);
  getstr("satmode", satmode);
  getstr( "f1180", f1180);
  getstr("cashape", cashape);
  getstr("prtinfo", prtinfo);

/* INITIALIZE VARIABLES */
  deltanh = ( (jnh > MIN_J) ? 1/(2*jnh) : 0.0 );
  deltacn = ( (jcn > MIN_J) ? 1/(2*jcn) : 0.0 );

/* LOAD PHASE TABLE */
  settable(t1, 4, phs1);
  settable(t2, 2, phs2);
  settable(t3, 2, phs3);
  settable(t4, 2, phs4);
  settable(t5, 2, phs5);
  settable(t6, 2, phs6);
  settable(t7, 64, phs7);

  setdivnfactor(t3, 32);
  setdivnfactor(t4, 4);
  setdivnfactor(t5, 16);
  setdivnfactor(t6, 8);

/* CHECK CONDITIONS */
  satmove = ( fabs(tof - satfrq) >= 0.1 );
  c13pwrmove = ( fabs(pwcolvl - pwcalvl) > 0.95 );
  exp3D = ( (int) (getvalnwarn("ni2") + 0.5) > 0 );
  if ( (n15dev != DODEV) && (n15dev != DO2DEV) )
  {
    text_error("invalid RF device for N15\n");
    abort(1);
  }
  else if ( (c13dev != DODEV) && (c13dev != DO2DEV) )
  {
    text_error("invalid RF device for C13\n");
    abort(1);
  }
  else if (c13dev == n15dev)
  {
    text_error("N15 and C13 RF devices must be different\n");
    abort(1);
  }
  if ( satmove && (satmode[C] == 'y') )
  {
    text_error("tof=satfrq is required for satmode[3]=='y'\n");
    abort(1);
  }
  dmn15 = ( (n15dev == DODEV) ? dm : dm2 );
  dmc13 = ( (c13dev == DODEV) ? dm : dm2 );
  if ( (strcmp(dmn15, "n") != 0) && (strcmp(dmn15, "nn") != 0) && (strcmp(dmn15, "ny") != 0) )
  {
    text_error("`dm(N15)` must be to 'n', 'nn', or 'ny'\n");
    abort(1);
  }
  if ( (strcmp(dmc13, "n") != 0) && (strcmp(dmc13, "nn") != 0) )
  {
    text_error("`dm(C13)` must be to 'n' or 'nn'\n");
    abort(1);
  }

/* DETERMINE STEADY-STATE MODE */
  if (ss < 0)
  {
    ss *= (-1);
```

```
      initval(ss, ssval);
      initval(ss, ssctr);
    }

 /* ADD IN STATES-HABERKORN ELEMENT */
    if (phase == 2)
      tsadd(t2, 1, 4);
    if (exp3D)
    {
      int      phase2;
      phase2 = (int) (getval("phase2") + 0.5);
      if (phase2 == 2)
        tssub(t1, 1, 4);
    }

 /* ADD IN FAD */
    if (fad1[A] == 'y')
    {
      if (ix == 1)
        d2_init = d2;
      t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
      if (t1_counter % 2)
      {
        tsadd(t2, 2, 4);              /* first C13 90 phase cycle    */
        tsadd(t7, 2, 4);              /* receiver phase cycle                    */
      }
    }
    if (exp3D)
    {
      char     fad2[MAXSTR];
      int      t2_counter;
      sw2 = getval("sw2");
      getstr("fad2", fad2);
      getstr("f2180", f2180);
      if (fad2[A] == 'y')
      {
        if (ix == 1)
          d3_init = d3;
        t2_counter = (int) ( (d3 - d3_init)*sw2 + 0.5 );
        if (t2_counter % 2)
        {
          tsadd(t1, 2, 4);       /* first N15 90 phase cycle    */
          tsadd(t7, 2, 4);      /* receiver phase cycle        */
        }
      }
    }

 /* BEGIN ACTUAL PULSE SEQUENCE CODE */
    status(A);
      rlpower(tpwr, TODEV);                /*  H1 hard-pulse power level*/
      rlpower(pwn15lvl, n15dev);           /* N15 hard-pulse power level              */
      rlpower(pwcolvl, c13dev);            /* CO pulse power              */
      offset(cofrq, c13dev);               /* CO frequency                            */
      if (sspul[A] == 'y')
      {
        rgpulse(200*pw, zero, rof1, 0.0);
        rgpulse(200*pw, one, 0.0, rof2);
      }
        hsdelay(d1);
 /* selective saturation period */
      if (satmode[A] == 'y')
      {
```

```
      if (satmove)
        offset(satfrq, TODEV);
      rlpower(satpwr, TODEV);
      rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
      if (satmove)
        offset(tof, TODEV);
      rlpower(tpwr, TODEV);
      delay(1.0e-5);
    }

/* Pulse train */
    rcvroff();
    decphase(t2);        /* C13 selective decoupling phase */
/* INEPT transfer from H1 to N15 */
    rgpulse(pw, zero, rof1, 5.0e-6);
    txphase(zero);                          /* H1 phase  */
    dec2phase(t1);                  /* N15 phase */
    delay(deltanh - 5.0e-6);
    sim3pulse(2*pw,0.0, pwn15, zero,zero, t1, 0.0, 5.0e-6);
    txphase(one);                           /* H1 phase  */
    dec2phase(t3);                  /* N15 phase */
    delay(deltanh - 5.0e-6);
    rgpulse(pw, one, 0.0, 0.0);
/* HMQC established between N15 and C13 */
    if (satmode[B] == 'y')
    {
      rlpower(satpwr, TODEV);
      txphase(zero);                        /* H1 phase for presaturation */
      if (satmove)
      {
        offset(satfrq, TODEV);
        rgpulse(deltacn - 2*(POWER_DELAY + OFFSET_DELAY) - deltanh - pw, zero, 0.0, 0.0);
        offset(tof, TODEV);
      }
      else
      {
        rgpulse(deltacn - 2*POWER_DELAY - deltanh - pw, zero, 0.0, 0.0);
      }
      ( (satmode[C] == 'y') ? delay(POWER_DELAY) : rlpower(tpwr, TODEV) );
    }
    else
    {
      delay(deltacn - deltanh - pw - POWER_DELAY);
      txphase(zero);
      ( (satmode[C] == 'y') ? rlpower(satpwr, TODEV) : delay(POWER_DELAY) );
    }
/* Calculate t1 evolution times */
    if (ix == 1)
    {
      t1adj_1 = (2*pwco/M_PI) + pwca + WFG_START_DELAY;
      t1adj_2 = pwn15 + pwca + WFG_STOP_DELAY;
      t1adj_3 = pwn15 + pwca + WFG_START_DELAY;
      t1adj_4 = (2*pwco/M_PI) + pwca + WFG_STOP_DELAY;
      if (c13pwrmove)
      {
        t1adj_1 += POWER_DELAY + 3.0e-6;
        t1adj_4 += POWER_DELAY + 3.0e-6;
      }
      t1evol_1 = (8 * sw1) * (MIN_DELAY + t1adj_1);
      t1evol_2 = (8 * sw1) * (MIN_DELAY + t1adj_2);
      t1evol_3 = (8 * sw1) * (MIN_DELAY + t1adj_3);
      t1evol_4 = (8 * sw1) * (MIN_DELAY + t1adj_4);
    tmpd2 = ( (t1evol_1 > t1evol_2) ? t1evol_1 : t1evol_2 );
    if (tmpd2 < t1evol_3)
      tmpd2 = t1evol_3;
    if (tmpd2 < t1evol_4)
      tmpd2 = t1evol_4;
    count = (int) (tmpd2) + 1;
    if (prtinfo[0] == 'y')
    {
      char  msge[128];
      double maxsw1;
      maxsw1 = ( (f1180[0] == 'y') ? (sw1/tmpd2) : (2*sw1)/tmpd2 );
      (void) sprintf(msge, "\nMaximum C13 spectral width = %d\n",
                                  (int) (maxsw1 + 0.5) );
      text_error(msge);
      (void) sprintf(msge, "count = %d\n", count);
      text_error(msge);
    }
    tmpd2 = count * (0.5/sw1);
  }
  if ( (f1180[0] != 'y') && (count < 3) )
  {
    t1evol_1 = (d2/4) - t1adj_1;
    t1evol_2 = (d2/4) - t1adj_2;
    t1evol_3 = (d2/4) - t1adj_3;
    t1evol_4 = (d2/4) - t1adj_4;
  }
  else
  {
    if ( (count > 2) || ((f1180[0] == 'y') && (count > 1)) )
    {
      if (ix == 1)
        text_error("WARNING:  sw1 is too large for proper t1 timing\n");
    }
    d2local = d2 + tmpd2;
    t1evol_1 = (d2local/4) - t1adj_1;
    t1evol_2 = (d2local/4) - t1adj_2;
    t1evol_3 = (d2local/4) - t1adj_3;
    t1evol_4 = (d2local/4) - t1adj_4;
  }
  if (t1evol_1 < MIN_DELAY)
    t1evol_1 = 0.0;
  if (t1evol_2 < MIN_DELAY)
    t1evol_2 = 0.0;
  if (t1evol_3 < MIN_DELAY)
    t1evol_3 = 0.0;
  if (t1evol_4 < MIN_DELAY)
    t1evol_4 = 0.0;
/* Start C13 pulsing */
  decrgpulse(pwco, t2, 0.0, 0.0);
  if (satmode[C] == 'y')
    xmtron();                   /* H1 presaturation          */
  if (c13pwrmove)
    rlpower(pwcalvl, c13dev);   /* Ca pulse power level                 */
  decphase(zero);        /* Ca phase                         */
  delay(t1evol_1);
  if (c13pwrmove)
    delay(3.0e-6);
  decshaped_pulse(cashape, 2*pwca, zero, 0.0, 0.0);
  delay(t1evol_2);
  decphase(zero);
  dec2rgpulse(2*pwn15, t3, 0.0, 0.0);
  delay(t1evol_3);
```

```
    decshaped_pulse(cashape, 2*pwca, zero, 0.0, 0.0);
    if (c13pwrmove)
      rlpower(pwcolvl, c13dev);    /* CO pulse power level          */
    decphase(t4);                  /* CO phase                      */
    dec2phase(zero);                         /* N15 phase                      */
    delay(t1evol_4);
    if (c13pwrmove)
      delay(3.0e-6);
    decrgpulse(pwco, t4, 0.0, 0.0);
    rlpower(pwcoalvl, c13dev);               /* C13 broadband pulse power      */
    decphase(zero);                /* C13 phase                     */
    if (satmode[C] == 'y')
      xmtroff();
/* Remove HMQC between N15 and C13 */
    delayval = deltacn - deltanh - 2*POWER_DELAY;
    if (exp3D)
      delayval -= 8*pwcoa;
    if (satmode[D] == 'y')
    {
      txphase(zero);                          /* H1 phase     */
      ( (satmode[C] == 'y') ? delay(POWER_DELAY) : rlpower(satpwr, TODEV) );
      if (satmove)
      {
        offset(satfrq, TODEV);
        rgpulse(delayval - POWER_DELAY - 2*OFFSET_DELAY, zero, 0.0, 0.0);
        offset(tof, TODEV);
      }
      else
      {
        rgpulse(delayval - POWER_DELAY, zero, 0.0, 0.0);
      }
      ( (exp3D) ? txphase(zero) : txphase(t5) );
      rlpower(tpwr, TODEV);
    }
    else
    {
      ( (exp3D) ? txphase(zero) : txphase(t5) );
      ( (satmode[C] == 'y') ? rlpower(tpwr, TODEV) : delay(POWER_DELAY) );
      delay(delayval);
    }
/* 3D section for N15 t2 evolution */
    if (exp3D)
    {
      double         t2evol;
      t2evol = d3;
      if (f2180[0] == 'y')
        t2evol += (0.5/sw2);
      delay(t2evol/2);
      decrgpulse(pwcoa, zero, 0.0, 0.0);
      simpulse(2*pw, 2*pwcoa, zero, one, 0.0, 0.0);
      decrgpulse(pwcoa, zero, 0.0, 0.0);
      txphase(t5);                           /* H1 phase         */
      delay(t2evol/2);
      decrgpulse(pwcoa, zero, 0.0, 0.0);
      decrgpulse(2*pwcoa, one, 0.0, 0.0);
      decrgpulse(pwcoa, zero, 0.0, 0.0);
    }
/* REVINEPT transfer from N15 back to H1 */
    rgpulse(pw, t5, 0.0, 5.0e-6);
    txphase(t6);                             /* H1 phase  */
    delay(deltanh - pw - 5.0e-6);
    sim3pulse(2*pw,0.0, pwn15, t6,zero, zero, 0.0, 0.0);
```

```
        rlpower(dpwr, DODEV);              /* N15 or C13 decoupling power */
        rlpower(dpwr2, DO2DEV);                      /* C13 or N15 decoupling power */
        dec2phase(zero);                   /* N15 decoupling phase */
        decphase(zero);           /* C13 final phase */
        delay(rof2);
        rcvron();
        delay(deltanh - 2*POWER_DELAY - PRG_START_DELAY - rof2);
      /* Start N15 broadband decoupling */
       status(B);
        setreceiver(t7);
}


      /* hncoct_sl.c  - HNCO 3D experiment with constant-time evolution in F2 (C=O) - 13C carrier (dec #1) on
      C=O and shifted pulse on Ca - ref.: Grzesiez and Bax, J. Magn. Reson., 96, 432-440 (1992).  - programmed
      by S.M. Gagne (6 July 1992) - last revised (13 August 1992)- added spin lock pulses C.M. Slupsky
      (February, 1993).

      Parameters:
              sspul = 'y':  selects for Trim(x)-Trim(y) sequence at the start of the pulse sequence
              f1180 = 'n':  standard t1 timing
                      'y':  modified t1 timing for t1(1) = half the dwell time
              f2180 = 'n':  standard t2 timing
                      'y':  modified t2 timing for t2(1) = half the dwell time
                      'n':  normal experiment
              fad1 = 'y':  TPPI axial-peak displacement along t1
                     'n':  standard phasecycle
              fad2 = 'y':  TPPI axial-peak displacement along t2 (3D experiment)
                     'n':  standard phasecycle
              satmode = 'y':  H1 presaturation during relaxtion delay
              satfrq = frequency of 1H presaturation for all periods
              satdly = saturation time during the relaxation period
              satpwr = saturation power for all periods of presaturation with xmtr
              hs = 'yn':  homospoil pulse (hst) during the d1 relaxation delay
              tpwr = power level for 1H transmitter pulses
              pw = 90 degree xmtr pulse length for protons (the observed nucleus)
              hard = hard shape-pulse for 1H and 15N
              dof = should be set to C0 frequency
              pwcalvl = power level for Ca decoupler pulses
              pwca180 = 180 degree decoupler pulse length for Ca at `pwcalvl`
              cashape = frequency-shifted pulse for 180 degree Ca excitation (see cashape note below)
              pwcolvl = power level for C0 decoupler pulses
              pwco = 90 degree decoupler pulse length for C0 at `pwcolvl`
              pwn15lvl = power level for N15 decoupler pulses
              pwn15 = 90 degree decoupler pulse length for N15
              dpwr2 = power level for N15 broadband decoupling
              inept = inept delay in sequence (1/4*NH scalar coupling) (about 2.45ms)
              tau2 = second delay in sequence ( about 3ms )
              tau1 = first delay in sequence ( about 10ms )
              dm2 = 'nnnnn':  no broadband decoupling of N15 during acquisition
                    'nnnny':  broadband heteronuclear decoupling of N15 during acquisition
              dm = 'n':  no broadband decoupling of C13 during acquisition
              nt = multiple of 16
              phase = 1,2:  hypercomplex experiment with F1 quadrature (complex F1-FT)
              phase2 = 1,2:  hypercomplex experiment with F2 quadrature (complex F2-FT)
              NOTES:  cashape :  the first null of the 180 Ca shifted pulse should correspond to the frequency of
              the C=O carrier (dof)
              constant time :        the constant-time evolution period is equal to 2*(tau1+tau2) and should be set
              to an odd multiple of 1/(2JNH) (~26 ms)
              Sensitivity : this experiment is a lot more sensitive then the HNCA or HN(CO)CA.  Less transients
              are necessary and since the spectral width in C=O is usually small, less increments in F2 (ni) are
              necessary.
      */
```

```
#include <standard.h>
#include <math.h>

#define MIN_DELAY           0.2e-6                /* shortest executable delay */
#define SIM3SH_START_DELAY 30.2e-6
#define SIM3SH_STOP_DELAY 12.2e-6

static int    phs1[2] = {0,2},
                  phs2[2] = {1,3},
                  phs3[1] = {0},
                  phs4[16]= {0,0,0,0,1,1,1,1,2,2,2,2,3,3,3,3},
                 phs5[4] = {0,0,2,2},
                 phs6[2] = {0,2},
                  rec[8] = {0,0,2,2,2,2,0,0};
static double        d2_init = 0.0,
                 d3_init = 0.0;

pulsesequence()
{
/* VARIABLE DECLARATION */
  char     sspul[MAXSTR],
                 fad1[MAXSTR],
                 fad2[MAXSTR],
                 f1180[MAXSTR],
                 f2180[MAXSTR],
                 hard[MAX STR],
                 spinlck[MAXSTR],
                 cashape[MAXSTR];
  int            phase,
                 satmove,
                 t1_counter,
                 t2_counter,
                 c13dev = DODEV,
                 n15dev = DO2DEV;
  double  ss,
                 t1evol,
                 t2evol_1,
                 t2evol_2,
                 ni2,
                 sltime1,
                 sltime2,
                 slpwr,
                 pwcalvl,
                 pwca180,
                 pwcolvl,
                 pwco,
                 pwn15lvl,
                 pwn15,
                 inept,
                 tau2,
                 tau1;


 /* Load variables */
   satfrq = getval("satfrq");
   satdly = getval("satdly");
   satpwr = getval("satpwr");
   pwcalvl = getval("pwcalvl");
   pwca180 = getval("pwca180");
   pwcolvl = getval("pwcolvl");
   pwco = getval("pwco");
```

```
    pwn15lvl = getval("pwn15lvl");
    pwn15 = getval("pwn15");
    inept = getval("inept");
    tau2 = getval("tau2");
    tau1 = getval("tau1");
    ss = getval("ss");
    sw1 = getval("sw1");
    sw2 = getval("sw2");
    ni2 = getval("ni2");
    sltime1 = getval("sltime1");
    sltime2 = getval("sltime2");
    slpwr = getval("slpwr");
    phase = (int) (getval("phase") + 0.5);
    phase2 = (int) (getval("phase2") + 0.5);

    getstr("spinlck",spinlck);
    getstr("cashape", cashape);
    getstr("hard", hard);
    getstr("sspul", sspul);
    getstr("fad1", fad1);
    getstr("fad2", fad2);
    getstr("satmode", satmode);
    getstr("f1180", f1180);
    getstr("f2180", f2180);

 /* Load phase cycles */
   settable(t1, 2, phs1);
   settable(t2, 2, phs2);
   settable(t3, 1, phs3);
   settable(t4, 16, phs4);
   settable(t5, 4, phs5);
   settable(t6, 2, phs6);
   settable(t7, 8, rec);

 /* Check conditions */
   satmove = ( fabs(tof - satfrq) >= 0.1 );
   if ( (dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y') )
   {
     text_error("`dm2' must be to 'nnnnn' or 'nnnny' for N15 decoupling\n");
     abort(1);
   }
   if ( (dm[A] == 'y') || (dm[B] == 'y') || (dm[C] == 'y') || (dm[D] == 'y') || (dm[E] == 'y') )
   {
     text_error("`dm' must be to 'n' for C13\n");
     abort(1);
   }
   if ( (tau2+tau1) <= ((ni2/sw2)/2) )
   {  text_error("`tau2+tau1' must be larger then `(ni2/sw2)/2'");
     abort(1);
   }

 /* Determine steady-state mode */
   if (ss < 0)
   {
     ss *= (-1);
     initval(ss, ssval);
     initval(ss, ssctr);
   }

 /* Add in States-Haberkorn element */
   if (phase2 == 2)                  /* N15 t1 element */
     tsadd(t3, 1, 4);
```

```
  if (phase == 2)                /* C13 t2 element */
    tsadd(t5, 1, 4);

/* Add in FAD */
  if (fad2[A] == 'y')            /* for N15 */
  {
    if (ix == 1)
      d3_init = d3;
    t2_counter = (int) ( (d3 - d3_init)*sw2 + 0.5 );
    if (t2_counter % 2)
    {
      tsadd(t3, 2, 4);           /* first N15 90-degree pulse   */
      tsadd(t7, 2, 4);           /* receiver phase cycle                    */
    }
  }
  if (fad1[A] == 'y')            /* for C13 */
  {
    if (ix == 1)
      d2_init = d2;
    t1_counter = (int) ( (d2 - d2_init)*sw1 + 0.5 );
    if (t1_counter % 2)
    {
      tsadd(t5, 2, 4);     /* first C13a 90-degree pulse   */
      tsadd(t7, 2, 4);     /* receiver phase cycle        */
    }
  }

/* BEGIN ACTUAL PULSE SEQUENCE CODE */
  status(A);
    rlpower(tpwr, TODEV);                /*  H1 hard-pulse power level */
    rlpower(pwn15lvl, n15dev);           /*  N15 hard-pulse power level              */
    rlpower(pwcalvl, c13dev);            /*  Ca 180 power                */
    if (sspul[A] == 'y')
    {
      rgpulse(200*pw, zero, rof1, 0.0);
      rgpulse(200*pw, one, 0.0, rof2);
    }
      hsdelay(d1);
/* selective saturation period */
    if (satmode[A] == 'y')
    {
      if (satmove)
        offset(satfrq, TODEV);
      rlpower(satpwr, TODEV);
      rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
      if (satmove)
        offset(tof, TODEV);
      rlpower(tpwr, TODEV);
      delay(1.0e-5);
    }

/* Pulse train */
  status(B);
    rcvroff();
    rgpulse(pw, zero, rof1, 0.0);
    dec2phase(t1);               /* N15 phase */
    decphase(zero);              /* C13 phase */
    delay(inept);
    sim3pulse(2*pw,0.0, 2*pwn15, zero,zero, t1, 0.0, 0.0);
    txphase(t2);                             /* H1 phase  */
    dec2phase(t3);               /* N15 phase */
    if (spinlck[0] == 'y')
  {
    rlpower(slpwr, TODEV);
    txphase(zero);
    delay(inept-POWER_DELAY);
    xmtron();
    delay(sltime1);
    xmtroff();
    rlpower(tpwr,TODEV);
    txphase(t2);
  }
  else
    delay(inept);
  sim3pulse(pw,0.0, pwn15, t2,zero, t3, 0.0, 0.0);
  txphase(zero);
  dec2phase(t4);
  t1evol = d3;      /* N15 is governed by ni2 and sw2 */
  if (f2180[0] == 'y')
    t1evol += 0.5/sw2;
  if (t1evol > MIN_DELAY)
  {
    t1evol -= pwn15 + pwca180 + 2*WFG_START_DELAY ;
    if (t1evol < MIN_DELAY)
      t1evol = 0.0;
  }
  delay(t1evol/2);
  decshaped_pulse(cashape,pwca180,zero,0.0,0.0);

status(C);
  delay( (tau1 + tau2)/2 - pwca180/2 - WFG_STOP_DELAY);
  rlpower(pwcolvl,c13dev);
  delay( (tau1 + tau2)/2 - POWER_DELAY );
  sim3pulse(2*pw, 2*pwco, 2*pwn15, zero, zero, t4,0.0,0.0);
  decphase(t5);
  dec2phase(t6);
  delay(tau1 +tau2-(t1evol/2+pwn15/2+pwca180/2+WFG_START_DELAY- pwco/2) );
  sim3pulse(0.0,pwco,pwn15, zero, t5, t6, 0.0, 0.0);
  rlpower(pwcalvl, c13dev);
  decphase(zero);
  dec2phase(zero);
  t2evol_1 = t2evol_2 = d2/2;          /* C13 is governed by ni and sw */
  if (f1180[0] == 'y')
  {
    t2evol_1 += 0.25/sw1;
    t2evol_2 += 0.25/sw1;
  }
  if ( (t2evol_1 > MIN_DELAY) || (t2evol_2 > MIN_DELAY) )
  {
    t2evol_1 -= pwco/2 + pwca180/2 + POWER_DELAY + SIM3SH_START_DELAY;
    t2evol_2 -= pwco/2 + pwca180/2 + POWER_DELAY + SIM3SH_STOP_DELAY;
    if (t2evol_1 < MIN_DELAY)
      t2evol_1 = 0.0;
    if (t2evol_2 < MIN_DELAY)
      t2evol_2 = 0.0;
  }
  delay(t2evol_1);
  sim3shaped_pulse(hard,cashape,hard,2*pw, pwca180, 2*pwn15, zero, zero, zero,0.0, 0.0);
  rlpower(pwcolvl, c13dev);
  delay(t2evol_2);

status(D);
  sim3pulse(0.0,pwco,pwn15,zero,zero, zero, 0.0, 0.0);
  delay(tau1 + tau2 - pwco/2);
```

```
      sim3pulse(0.0,2*pwco,2*pwn15,zero, zero, zero, 0.0, 0.0);
      delay(tau1 - pw);
      rgpulse(2*pw, zero, 0.0, 0.0);
      delay(tau2 - pw -pwn15/2);
      sim3pulse(pw,0.0, pwn15, zero ,zero, zero, 0.0, 0.0);
      delay(inept);
      sim3pulse(2*pw,0.0, 2*pwn15, zero, zero,zero, 0.0, 0.0);
      rlpower( ((n15dev == DODEV) ? dpwr : dpwr2), n15dev);
      rlpower( ((c13dev == DODEV) ? dpwr : dpwr2), c13dev);
      if (spinlck[0] == 'y')
      {
        rlpower(slpwr,TODEV);
        txphase(zero);
        delay(inept-3*POWER_DELAY);
        xmtron();
        delay(sltime2);
        xmtroff();
        rlpower(tpwr,TODEV);
      }
      else
        delay(inept - rof2 - 2*POWER_DELAY);
      delay(rof2);
      rcvron();

    status(E);
      setreceiver(t7);
}
```

7. 3D HCANH experiment

```
/*hcannh1.c - The H(CA)NNH experiment by Kay et al. (J. Mag. Reson. 91 84-92). Uses frequency shifted
laminar pulses (Patt J. Mag. Reson. 96 94-102) to excite the Ca spins while on resonance with the CO spins.
Decoupling of the water is achieved by two spin lock sequences of approx. 1.5ms and 9ms. The delays may
be approximated as follows: tau1~(1/4Jch), tau2~(1/4Jch), tau4~(1/8Jnc) tau5~(1/8Jnc), tau3~(1/4Jnh),
tau~(1/4Jnh).
*/
/* CMS April 30,1992 */

/* Parameters:
        fad1 = 'y': TPPI axial-peak displacement along t1.
               'n': standard phase cycle.
        fad2 = 'y': TPPI axial-peak displacement along t2.
               'n': standard phase cycle.
        satpwr = power level for spin lock water.
        h1shape = proton 90 deg. hard pulse (specified due to sim3pulse).
        tpwr = transmitter power level for a normal proton pulse.
        pw = 90 deg. transmitter pulse length for protons.
        pwsat = 90 deg. transmitter pulse length for spinlock at satpwr.
        pwcalvl = power level for Ca decoupler pulses on DODEV.
        pwca = 90 deg. decoupler pulse length for Ca.
        cafrq = frequency of Ca spins.
        ca90shape = pulse pattern for generating a 90 deg shifted laminar pulse on DODEV.
        ca180shape = pulse pattern for generating a 180 deg shifted laminar pulse on DODEV.
        pwcolvl = power level for CO decoupling on DODEV.
        pwco = 90 deg. decoupler pulse length for pwcolvl.
        cores = tip angle resolution.
        coshape = decoupling pattern for CO decoupling.
        cofrq = frequency of CO spins.
        pwn15lvl = power level for N15 pulsing on DO2DEV.
        pwn15 = 90 deg decoupler pulse length for n15.
```

```
                n15shape = n15 90 deg. hard pulse (specified for sim3pulse).
                dm2 = 'nny' = decoupling of n15 during acquisition at dpwr2.
                dpwr2 = dcoupling power of n15 (during acquisition).
                dm = 'nnn' = no decoupling of c13 during entire experiment.
*/

# include <standard.h>
# include <math.h>
# define  MIN_DELAY    0.2e-6
static double       d2_init = 0.0,
                d3_init = 0.0;
static int         phs1[1] = {0},
                phs2[2] = {0, 2},
                phs3[2] = {1, 3},
                phs4[4] = {0, 0, 2, 2},
                phs5[16]= {0, 0, 0, 0, 1, 1, 1, 1,
                           2, 2, 2, 2, 3, 3, 3, 3},
                phs6[32]= {0, 0, 0, 0, 0, 0, 0, 0,
                           0, 0, 0, 0, 0, 0, 0, 0,
                           2, 2, 2, 2, 2, 2, 2, 2,
                           2, 2, 2, 2, 2, 2, 2, 2},
                phs7[16]= {0, 0, 0, 0, 0, 0, 0, 0,
                           2, 2, 2, 2, 2, 2, 2, 2},
                phs8[32]= {0, 2, 2, 0, 2, 0, 0, 2,
                           0, 2, 2, 0, 2, 0, 0, 2,
                           2, 0, 0, 2, 0, 2, 2, 0,
                           2, 0, 0, 2, 0, 2, 2, 0};

/*------------------------------------------
|                                          |
|       pulsesequence()/0                  |
|                                          |
+-----------------------------------------*/

pulsesequence()
{

/*Variable Declaration*/

char    ca90shape[MAXSTR],
                                  ca180shape[MAXSTR],
      h1shape[MAXSTR],
      n15shape[MAXSTR],
      satflg[MAXSTR],
      fad1[MAXSTR],
      fad2[MAXSTR],
      sspul[MAXSTR];

int     phase,
      t1_counter,
      t2_counter;

double  ss,
      t1evol,
      t2evol,
      pwcolvl,
      pwcalvl,
      pwco,
      pwca,
      cores,
      cofrq,
      pwn15,
        pwn15lvl,
        tau1,
        tau2,
        tau3,
        tau4,
        tau5;

/*Load Variables*/

       satfrq=getval("satfrq");
       pwn15lvl=getval("pwn15lvl");
       pwcolvl=getval("pwcolvl");
       pwcalvl=getval("pwcalvl");
       pwca=getval("pwca");
       pwn15=getval("pwn15");
       satdly=getval("satdly");
       cofrq=getval("cofrq");
       cores=getval("cores");
       pwco=getval("pwco");
       ss=getval("ss");
       sw1=getval( "sw1");
       sw2=getval("sw2");
       tau1=getval("tau1");
       tau2=getval("tau2");
       tau3=getval("tau3");
       tau=getval("tau");
       tau4=getval("tau4");
       tau5=getval("tau5");
       satpwr=getval("satpwr");
       phase=(int)(getval("phase")+0.5);
       phase2=(int)(getval("phase2")+0.5);
       getstr("ca90shape",ca90shape);
       getstr("ca180shape",ca180shape);
       getstr("h1shape",h1shape);
       getstr("n15shape",n15shape);
       getstr("sspul",sspul);
       getstr("fad1",fad1);
       getstr("fad2",fad2);
       getstr("satflg",satflg);

/* Load Phase Cycles */

       settable(t1,1,phs1);
       settable(t2,2,phs2);
       settable(t3,2,phs3);
       settable(t4,4,phs4);
       settable(t5,16,phs5);
       settable(t6,32,phs6);
       settable(t7,16,phs7);
       settable(t8,32,phs8);

/* SAFETY CHECKS */

    if ((dm2[A] == 'y') || (dm2[B] == 'y') )
    {
      text_error("`dm2` must be set to 'nnn' or 'nny'\n");
      abort(1);
    }
    if ((dm[A] == 'y') || (dm[B] == 'y') || (dm[C] == 'y') )
    {
      text_error("`dm` must be set to 'nnn'\n");
      abort(1);
    }

  /* Determine Steady state mode */
    if (ss < 0)
    {
     ss *= (-1);
     initval(ss, ssval);
     initval(ss, ssctr);
    }

  /* ADD in FAD */
    if (fad1[A] == 'y')
    {
     if (ix == 1)
      d2_init=d2;
     t1_counter=(int)((d2-d2_init)*sw1+0.5);
     if (t1_counter %2)
      {
      tsadd(t1, 2, 4);
      tsadd(t8, 2, 4);
      }
    }
    if (fad2[A] == 'y')
    {
     if (ix == 1)
      d3_init=d3;
     t2_counter=(int)((d3-d3_init)*sw2+0.5);
     if (t2_counter %2)
      {
      tsadd(t6, 2, 4);
      tsadd(t8, 2, 4);
      }
    }

  /* ADD IN STATES-HABERKORN-RUBEN ELEMENT */

   if (phase == 2)
    tsadd(t1, 1, 4);
   if (phase2 == 2)
    tsadd(t6, 1, 4);

   status(A);          /* HCa evolution */
    rlpower(tpwr,TODEV);
   rlpower(pwn15lvl,DO2DEV);
   rlpower(pwcalvl,DODEV);
   offset(cofrq,DODEV);
   if (satflg[A] == 'y')
   {
    offset(s atfrq, TODEV);
    rlpower(satpwr,TODEV);
    rgpulse(satdly,zero,4.0e-5,0.2e-6);
    offset(tof,TODEV);
    rlpower(tpwr,TODEV);
    delay(1.0e-5);
   }

  status(B);
   rcvroff();
   txphase(t1);
   decphase(t2);
   t1evol=d2;
   if (t1evol > MIN_DELAY)
```

```
 {
 tlevol -= (2*pwca+WFG_START_DELAY+WFG_STOP_DELAY);
 if (tlevol < MIN_DELAY)
   tlevol=0.0;
 }
 rgpulse(pw,t1,1.0e-6,0.0);
 delay(tlevol/2);
 delay(tau1);
 txphase(zero);
 decshaped_pulse(ca180shape,2*pwca,t2,0.0,0.0);
 delay(tlevol/2);
 rgpulse(2*pw,zero,0.0, 0.0);
 delay(tau1);
/* transfer of magnetization to Ca and N  */
 txphase(t3);
 decphase(t4);
 simshaped_pulse(h1shape,ca90shape,pw,pwca,t3,t4,0.0,0.0);
 rlpower(pwcolvl,DODEV);
 decprgon(dseq,pwco,cores);
 decon();
 txphase(zero);
 delay(tau2-pw);
 rgpulse(2*pw,zero,0.0,0.0);
 dec2phase(zero);
 decphase(t5);
 delay(tau4-tau2-pw);
 decoff();
 decprgoff();
 rlpower(pwcalvl,DODEV);
sim3shaped_pulse(h1shape,ca180shape,n15shape,0.0,2*pwca,2*pwn15,zero,t5,zero,0.0,0.0);
 rlpower(pwcolvl,DODEV);
 decprgon(dseq,pwco,cores);
 decon();
 delay(tau4);
 decphase(zero);
 decoff();
 decprgoff();
 rlpower(pwcalvl,DODEV);
 decshaped_pulse(ca90shape,pwca,zero,0.0,0.0);
 rlpower(pwcolvl,DODEV);
 decprgon(dseq,pwco,cores);
 decon();
/* Evolution of 15N */
 txphase(one);
 dec2phase(t6);
 dec2rgpulse(pwn15,t6,0.0,0.0);
 txphase(zero);
 decphase(t7);
 dec2phase(zero);
 t2evol=d3;
 if(t2evol < MIN_DELAY)
   t2evol=0.0;
 delay(t2evol/2);
 delay(tau3-2*pw);
 rgpulse(2*pw,zero,0.0,0.0);
 delay(tau5-tau3-2*pwca);
 decoff();
 decprgoff();
 rlpower(pwcalvl,DODEV);
 decshaped_pulse(ca180shape,2*pwca,t7,0.0,0.0);
 rlpower(pwcolvl,DODEV);
 decprgon(dseq,pwco,cores);
```

```
    decon();
    delay(t2evol/2);
    dec2rgpulse(2*pwn15,zero,0.0,0.0);
    delay(tau5);
    decoff();
    decprgoff();
    rlpower(pwcalvl,DODEV);
   /* RevInept to NH */
    sim3pulse(pw,0.0,pwn15,zero,zero,zero,0.0,0.0);
    delay(tau);
    sim3pulse(2*pw,0.0,2*pwn15,zero,zero,zero,0.0,0.0);
    rlpower(dpwr2,DO2DEV);
    dec2phase(zero);
   delay(6.6e-6);
    rcvron();
    delay(tau-POWER_DELAY-6.6e-6);
    /* Start broadband 15N decoupling */
   status(C);
    setreceiver(t8);
   }
```

8. 3D HCCH-COSY experiment

```
/*hcchcosy.c - The HCCH-COSY experiment by Ikura et al. (J. Biol. NMR 1 299-304 (1991)). Uses constant
time evolution of the t2 dimension (13C) magnetization thereby optimizing the 13C-13C magnetization
transfer independent of t2 and removing the multiplet structure in the 13C dimension. Frequency shifted
pulses are used to simultaneously decouple carbonyl and aromatic carbon resonances. This was
accomplished by creating the pulse 'coar180' which is described by a sin(x)/x function giving approximately
180 degree pulses at approximately 134 ppm and 90 ppm from the carrier (See CMS Pulse Sequence Binder).
*/
/* CMS June 29, 1992 */

/* Parameters:

        constT = constant time parameter ~ 1/4Jcc = 2constT. A value for 2constT longer than 1/4Jcc
        decreases sensitivity.
         tau1 = 1.6 ms = 1/4Jhc
         tau2 = 1.1 ms To maximize magnetizetion transfer simultaneously for methine, methylene amd
        methyl carbons, this value was chosen ~ 0.3/JIS. If you want to suppress magnetization transfer to or
        from methylene or methyl sites, a longer value can be used which would increase sensitivity for the
        selected methine resonances.
         tau3 = 0.85 ms
         fad1 = 'n': standard phase cycle.
              = 'y': TPPI axial-peak displacement along t1.
         fad2 = 'n': standard phase cycle.
              = 'y': TPPI axial-peak displacement along t2.
         satflg = 'ynn': presaturation of the HDO.
         satfrq = frequency of the presaturation.
         satdly = saturation time during the relaxation period.
         satpwr = saturation power for presaturation of HDO.
         tpwr = transmitter power for all proton pulses (other than presaturation).
         pw = 90 degree pulse length for protons.
         cafrq = frequency for carbon pulses.
         pwcalvl = power for carbon pulses.
         pwca = 90 degree pulse length for carbon.
         coshape = shape of carbonyl decoupling.
*/

# include <standard.h>
# include <math.h>
# define MIN_DELAY     0.2e-6
# define SHPSTART_DELAY  10.2e-6
# define SHPSTOP_DELAY    4.2e-6
# define POWER_DELAY   4.2e-6

static double   d2_init = 0.0,
            d3_init=0.0;

static int   phs1[1] = {0};
         phs2[16] = {0,0,0,0,0,0,0,0,
                  2,2,2,2,2,2,2,2},
         phs3[16] = {0,0,0,0,1,1,1,1,
                  2,2,2,2,3,3,3,3},
         phs4[2] = {1,3},
         phs5[1] = {0},
         phs6[8] = {0,0,1,1,2,2,3,3},
         phs7[8] = {0,0,0,0,2,2,2,2},
         phs8[16] = {0,2,2,0,2,0,0,2,
                  2,0,0,2,0,2,2,0},
         phs9[8] = {0,0,0,0,2,2,2,2};

/*----------------------------------------------------
|                                                    |
|           pulsesequence()/0                        |
|                                                    |
+----------------------------------------------------*/

pulsesequence()
{

/* Variable Declaration */

  char   coshape[MAXSTR],
      hshape[MAXSTR],
      fad1[MAXSTR],
      fad2[MAXSTR],
      satmode[MAXSTR];

  int    phase,
      phase2,
      t1_counter,
      t2_counter;

  double ss,
      sw1,
      sw2,
      t1evol,
      t2evol,
      satpwr,
      pwcalvl,
      pwco,
      pwca,
      cafrq,
      satfrq,
      satdly,
      del1,
      del2,
      del3,
      del4,
      tau1,
      tau2,
          tau3,
          tau_2,
          tau_3,
          constT,
          constT1,
          constT2,
          ni2,
          minsw2;

/* Load Variables */

        satfrq = getval("satfrq");
        satdly = getval("satdly");
        satpwr = getval("satpwr");
        pwcalvl = getval("pwcalvl");
        tau1 = getval("tau1");
        tau2 = getval("tau2");
        tau3 = getval("tau3");
        pwco = getval("pwco");
        pwca = getval("pwca");
        cafrq = getval("cafrq");
        constT = getval("constT");
        ss = getval("ss");
        sw1 = getval("sw1");
        sw2 = getval("sw2");
        ni2 = getval("ni2");
        phase = (int)(getval("phase")+0.5);
        phase2 = (int)(getval("phase2")+0.5);
        getstr("coshape",coshape);
        getstr("hshape",hshape);
        getstr("fad1",fad1);
        getstr("fad2",fad2);
        getstr("satmode",satmode);

/* Load Phase Cycles */
        settable(t1, 1, phs1);
        settable(t2, 16, phs2);
        settable(t3, 16, phs3);
        settable(t4, 2, phs4);
        settable(t5, 1, phs5);
        settable(t6, 8, phs6);
        settable(t7, 8, phs7);
        settable(t8, 16, phs8);
        settable(t9, 8, phs9);

/* Safety Checks */
   if ((dm[A] == 'y' || dm[B] == 'y'))
     {
      text_error("dm should be set to 'nny'\n");
      abort(1);
     }
    if ((dm2[A] == 'y' || dm2[B] == 'y' || dm2[C] == 'y'))
     {
      text_error("dm2 should be set to 'nnn'\n");
      abort(1);
     }
    if (pw > 30)
     {
      text_error("pw is too large!!!");
      abort(1);
     }
    if (satpwr > 10)
    {
     text_error("satpwr is too large!!!");
     abort(1);
    }
    if (pwca > 50)
    {
     text_error("pwca is too large!!!");
     abort(1);
    }
    if (pwco > 50)
    {
     text_error("pwco is too large!!!");
     abort(1);
    }
    if (dpwr > 40)
    {
     text_error("dpwr is too large....you will wreck the probe!!!");
     abort(1);
    }
    if (pwcalvl > 55)
    {
     text_error("pwcalvl is too large...you will wreck the probe!!!");
     abort(1);
    }
    if (tpwr > 60)
    {
     text_error("tpwr is too large...you will wreck the probe!!!");
     abort(1);
    }

/* Error Checks */
  minsw2 = ((ni2-1)/constT)*1000;
  if (minsw2 < sw2)
  {
   text_error("sw2 is too small");
   abort(1);
  }

/* Determine Steady state mode */
    if (ss<0)
    {
     ss *= (-1);
     initval(ss, ssval);
     initval(ss, ssctr);
    }

/* Add in FAD */
    if (fad2[A] == 'y')
    {
     if (ix == 1)
       d3_init = d3;
     t2_counter = (int) ((d3 - d3_init)*sw2 + 0.5);
     if (t2_counter % 2 )
     {
      tsadd(t5, 2, 4);
      tsadd(t8, 2, 4);
     }
    }
    if (fad1[A] == 'y')
    {
     if (ix == 1)
       d2_init = d2;
    t1_counter = (int) ((d2 - d2_init)*sw1 + 0.5);
    if (t1_counter % 2)
    {
     tsadd(t1, 2, 4);
     tsadd(t8, 2, 4);
    }
   }

/* Add in States-Haberkorn Element */
   if (phase == 2)
    tsadd(t1, 1, 4);
   if (phase2 == 2)
    tsadd(t5, 1, 4);

status(A);
 rlpower(satpwr, TODEV);
 rlpower(pwcalvl, DODEV);
 offset(cafrq, DODEV);
 offset(satfrq, TODEV);
 if (satmode[A] == 'y')
  rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
 rcvroff();
 delay(POWER_DELAY);

status(B);            /* Begin Pulse Train */
 rlpower(tpwr, TODEV);
 offset(tof, TODEV);
 txphase(t1);
 decphase(t2);
 t1evol=d2;           /* Evolution of protons */
 if (t1evol > MIN_DELAY)
  t1evol -= (2*pwca);
 if (t1evol < MIN_DELAY)
  t1evol = d2;
 rgpulse(pw,t1,0.0,0.0);
 delay(tau1);
 delay(t1evol/2);
 decrgpulse(2*pwca,t2,0.0,0.0);
 txphase(t3);
 delay(t1evol/2);
 rgpulse(2*pw,t3,0.0,0.0);
 txphase(t4);
 decphase(t5);
 delay(tau1);
 simpulse(pw,pwca,t4,t5,0.0,0.0);
 txphase(zero);
 t2evol = d3;            /* Begin constant time evolution of carbon */
 delay(t2evol/4);
 delay(tau3-pw);
 rgpulse(2*pw,zero,0.0,0.0);
 decphase(zero);
 tau_2 = tau2-SHPSTART_DELAY-pwco-pw;
 delay(tau_2);
 decshaped_pulse(coshape,2*pwco,zero,0.0,0.0);
 decphase(t6);
 del1=tau3+tau2+(t2evol/4)-2*pwca-pwco-SHPSTOP_DELAY;
 delay(del1);
 decrgpulse(2*pwca,t6,0.0,0.0);
 txphase(t3);
 decphase(t2);
 constT1 = (((constT - (t2evol/2))/2) - SHPSTART_DELAY - pwco);
 constT2 = (((constT - (t2evol/2))/2) - SHPSTOP_DELAY -pwco);
```

```
    delay(constT1);
    simshaped_pulse(hshape,coshape,2*pw,2*pwco,t3,t2,0.0,0.0);
    decphase(t7);
    delay(constT2);
    decrgpulse(pwca,t7,0.0,0.0);
    decphase(zero);
    del2=tau2+tau3-SHPSTART_DELAY-pwco;
    del3=tau2+tau3-SHPSTOP_DELAY-pwco;
    delay(del2);
    decshaped_pulse(coshape,2*pwco,zero,0.0,0.0);
    delay(del3);
    decrgpulse(2*pwca,zero,0.0,0.0);
    decphase(t2);
    delay(del2);
    decshaped_pulse(coshape,2*pwco,t2,0.0,0.0);
    txphase(zero);
    tau_3=tau3-SHPSTOP_DELAY-pwco-pw;
    delay(tau_3);
    rgpulse(2*pw,zero,0.0,0.0);
    txphase(t2);
    decphase(zero);
    del4 = tau2 - pw;
    delay(del4);              /* End of constant time evolution of carbon */
    simpulse(pw,pwca,t2,zero,0.0,0.0);
    txphase(zero);
    decphase(t7);
    delay(tau1);
    simpulse(2*pw,2*pwca,zero,t7,0.0,0.0);
    delay(tau1);
    rcvron();
    decrgpulse(pwca, zero, 0.0, 0.0);
    decrgpulse(pwca, t9, 0.0, 0.0);
    rlpower(dpwr, DODEV);
    delay(POWER_DELAY);

  status(C);
    setreceiver(t8);
  }
```

9. 4D ^{13}C-^{15}N-edited NOESY experiment

```
/* nosyhmqc4d3rf - NOESY-HMQC 4D sequence - written in hypercomplex phase sensitive mode only -
uses the 2nd decoupler for the X pulses - f1=C, f2=H, f3=N, f4=H

 Parameters:
        d2 = First evolution time
        d3 = Second evolution time
        d4 = Third evolution time
        mix = NOESY mixing time.
        pwx2lvl = power level for 15N pulses
        pwxlvl = power level for 13C pulses
        pwx = 90 degree 13C pulse
        pwx2  = 90 degree 15N pulse
        pw = 90 degree H pulse
        delta_1 = 13C-H coupling constant set to slightly less than (1/2JHC) 3.0 ms
        delta_2 = 15N-H coupling constant set to slightly less than (1/2JHN) 4.5 ms
        dpwr2  = power level for 15N decoupling
        tpwr   = power level for H pulses
        phase  =   1,2: gives HYPERCOMPLEX (t1) acquisition;
        ni     = number of t1 increments
```

```
            phase2 =  1,2: gives HYPERCOMPLEX (t2) acquisition;
            ni2  = number of t2 increments
            phase3 =  1,2: gives HYPERCOMPLEX (t3) acquisition;
            ni3  = number of t3 increments
            satflg  = flag to do xmtr presaturation at satfrq and satpwr during relaxation (satdly) and mixing (mix)
              periods
            satfrq  = saturation frequency for xmtr presaturation
            satpwr  = saturation power for xmtr presaturation
            satdly  = saturation period follows D1
            sspul = 'y': selects for saturation sequence at start of pulse sequence

            phase table:
                t1 = 0 0 0 0  - first proton pulse
                t2 = 0 2 0 2  - first carbon pulse
                t3 = 0 0 2 2  - first nitrogen pulse
                t4 = 0 2 2 0  - receiver

June 22, 1994 (Carolyn Slupsky)  */

#include <standard.h>
#include <math.h>

#define MIN_DELAY   0.2e-6     /* shortest executable delay */

static int    phs1[4] = {0,0,0,0},
                              phs2[4] = {0,2,0,2},
                              phs3[4] = {0,0,2,2},
                              phs4[4] = {0,2,2,0};

static double  d2_init = 0.0,
                              d3_init = 0.0,
                              d4_init = 0.0;

pulsesequence()
{
/* VARIABLE DECLARATION */
  double  ss,
                   t1evol_1,
                   t2evol_1,
                   t3evol_1,
                   t1evol_2,
                   t2evol_2,
                   t3evol_2,
                   mix,
                   delta_1,
                   delta_2,
                   pwx2lvl;

  int              phase,
                   satmove,
                   t1_counter,
                   t2_counter,
                   t3_counter;

  char             sspul[MAXSTR],
                   satflg[MAXSTR],
                   fad1[MAXSTR],
                   fad2[MAXSTR],
                   fad3[MAXSTR],
                   f1180[MAXSTR],
                   f2180[MAXSTR],
                   f3180[MAXSTR];
```

```
/* LOAD VARIABLES */
 satdly = getval("satdly");
 satfrq = getval("satfrq");
 satpwr = getval("satpwr");
 ss = getval("ss");
 mix = getval("mix");
 pwx2lvl = getval("pwx2lvl");
 pwxlvl = getval("pwxlvl");
 pwx2 = getval("pwx2");
 pwx = getval("pwx");
 sw1 = getval("sw1");
 sw2 = getval("sw2");
 sw3 = getval("sw3");
 delta_1 = getval("delta_1");
 delta_2 = getval("delta_2");
 phase = (int) (getval("phase") + 0.5);
 phase2 = (int) (getval("phase2") + 0.5);
 phase3 = (int) (getval("phase3") + 0.5);

  getstr("sspul", sspul);
  getstr("satflg",satflg);
  getstr("fad1",fad1);
  getstr("fad2",fad2);
  getstr("fad3",fad3);
  getstr("f1180",f1180);
  getstr("f2180",f2180);
  getstr("f3180",f3180);

/* LOAD PHASE CYCLES */
  settable(t1, 4, phs1);
  settable(t2, 4, phs2);
  settable(t3, 4, phs3);
  settable(t4, 4, phs4);

/* CHECK CONDITIONS */
          satmove = (fabs(tof - satfrq) >= 0.1);
  if ((dm2[A] == 'y') || (dm2[B] == 'y') || (dm2[C] == 'y') || (dm2[D] == 'y') || (dm2[E] == 'y'))
  {
   text_error("'dm2' must be 'nnnnnn' or 'nnnnny' for N15 decoupling\n");
   abort(1);
  }

  if ((dm[A] == 'y') || (dm[B] == 'y') || (dm[C] == 'y') || (dm[D] == 'y') || (dm[E] == 'y') || (dm[F] == 'y'))
          {
           text_error("'dm' must be 'n' for C13 \n");
           abort(1);
  }

/* DETERMINE STEADY-STATE MODE */
  if (ss < 0)
          {
           ss *= (-1);
           initval(ss, ssval);
           initval(ss, ssctr);
  }

/* Add in States-Haberkorn element */
  if (phase == 2)   /* C13 t1 element */
           tsadd(t2, 1, 4);
          if (phase2 == 2)  /* 1H t2 element */
           tsadd(t1, 1, 4);
   if (phase3 == 2)  /* 15N t3 element */
          tsadd(t3, 1, 4);

/* Add in FAD */
          if (fad1[A] == 'y')     /* for 13C */
          {
            if (ix == 1)
            d2_init = d2;
            t1_counter = (int) ((d2 - d2_init)*sw1 + 0.5);
            if (t1_counter % 2)
            {
                      tsadd(t2, 2, 4);  /* first 13C 90-degree pulse */
                      tsadd(t4, 2, 4);  /* receiver phase cycle */
            }
          }

          if (fad2[A] == 'y')     /* for 1H */
          {
            if (ix == 1)
            d3_init = d3;
            t2_counter = (int) ((d3 - d3_init)*sw2 + 0.5);
            if (t2_counter % 2)
            {
                      tsadd(t1, 2, 4);  /* first 1H 90-degree pulse */
                      tsadd(t4, 2, 4);  /* receiver phase cycle */
            }
          }
          if (fad3[A] == 'y')     /* for 15N */
          {
            if (ix == 1)
            d4_init = d4;
            t3_counter = (int) ((d4 - d4_init)*sw3 + 0.5);
            if (t3_counter % 2)
            {
                      tsadd(t3, 2, 4);  /* first 1H 90-degree pulse */
                      tsadd(t4, 2, 4);  /* receiver phase cycle */
            }
          }

/* BEGIN THE ACTUAL PULSE SEQUENCE */
  status(A);              /* relaxation status */
  rlpower(tpwr,TODEV);
  rlpower(pwxlvl,DODEV);
  rlpower(pwx2lvl,DO2DEV);
  decphase(t2);    /* 13C phase */
  dec2phase(t3);   /* 15N phase */
  if (sspul[A] == 'y')
  {
    rgpulse(pw*200, zero, rof1,rof2);
    rgpulse(pw*200, one, rof1,rof2);
  }
  else
    hsdelay(d1);
  if (satflg[A] == 'y')   /* selective saturation period */
   {
     if (satmove)
      offset(satfrq,TODEV);
     rlpower(satpwr,TODEV);
     rgpulse(satdly, zero, 4.0e-5, 0.2e-6);
     rlpower(tpwr,TODEV);
     if (satmove)
      offset(tof,TODEV);
   delay(0.00004);
  }
else
 delay(satdly);

status(B);          /* Pulse train */
        rcvroff();
        txphase(t1);    /* 1H phase */
  rgpulse(pw,t1,0.0,0.0);
        txphase(zero);
        delay(delta_1 + 2.0*pwx);
        decrgpulse(pwx,t2,0.0,0.0);
        decphase(zero);
        t1evol_1 = d2/2;  /* C13 is governed by ni, and sw1 */
        t1evol_2 = d2/2;
        if (f1180[0] == 'y')
        {
                t1evol_1 += 0.25/sw1;
                t1evol_2 += 0.25/sw1;
        }
        if ((t1evol_1 > MIN_DELAY) || (t1evol_2 > MIN_DELAY))
        {
                t1evol_1 -= pw + 1.15e-6;
                t1evol_2 -= pw;
                if (t1evol_1 < MIN_DELAY)
                        t1evol_1 = 0.0;
    if (t1evol_2 < MIN_DELAY)
                        t1evol_2 = 0.0;
        }

        delay(t1evol_1);
        rgpulse(2*pw,zero,0.0,0.0);
        delay(t1evol_2);
        decrgpulse(pwx,zero,0.0,0.0);
        delay(delta_1);

 status(C);
        t2evol_1 = d3/2;   /* 1H is governed by ni2 and sw2 */
        t2evol_2 = d3/2;
        if (f2180[0] == 'y')
        {
                t2evol_1 += 0.25/sw2;
                t2evol_2 += 0.25/sw2;
        }
        if ((t2evol_1 > MIN_DELAY) || (t2evol_2 > MIN_DELAY))
        {
                t2evol_1 -= pwx;
                t2evol_2 -= pwx;
                if (t2evol_1 < MIN_DELAY)
                        t2evol_1 = 0.0;
    if (t2evol_2 < MIN_DELAY)
                        t2evol_2 = 0.0;
        }
   delay(t2evol_1);
         decrgpulse(2*pwx,zero,0.0,0.0);
         delay(t2evol_2);
         rgpulse(pw,zero,0.0,0.0);

 status(D);
         if (satflg[D] == 'y')
         {
                if (satmove)
```

```
                        offset(satfrq, TODEV);
      rlpower(satpwr, TODEV);
                       rgpulse(mix,zero,2.0e-6,rof1);
                       rlpower(tpwr,TODEV);
                       if (satmove)
                        offset(tof, TODEV);
      delay(0.000040);
             }
             else
              hsdelay(mix);

   status(E);
             rgpulse(pw,zero,0.0,0.0);
    delay(delta_2);
             dec2rgpulse(pwx2,t3,0.0,0.0);
             dec2phase(zero);
            t3evol_1 = d4/2;     /* 15N is governed by ni3 and sw3 */
            t3evol_2 = d4/2;
            if (f3180[0] == 'y')
            {
                      t3evol_1 += 0.25/sw3;
                      t3evol_2 += 0.25/sw3;
            }
            if ((t3evol_1 > MIN_DELAY) || (t3evol_2 > MIN_DELAY))
            {
                      t3evol_1 -= pw + 1.15e-6;
                      t3evol_2 -= pw;
                      if (t3evol_1 < MIN_DELAY)
                                t3evol_1 = 0.0;
     if (t3evol_2 < MIN_DELAY)
                                t3evol_2 = 0.0;
            }
            delay(t3evol_1);
            rgpulse(2*pw,zero,0.0,0.0);
            delay(t3evol_2);
            dec2rgpulse(pwx2,zero,0.0,0.0);
            rcvron();
            rlpower(dpwr2, DO2DEV);
            delay(delta_2 - POWER_DELAY);

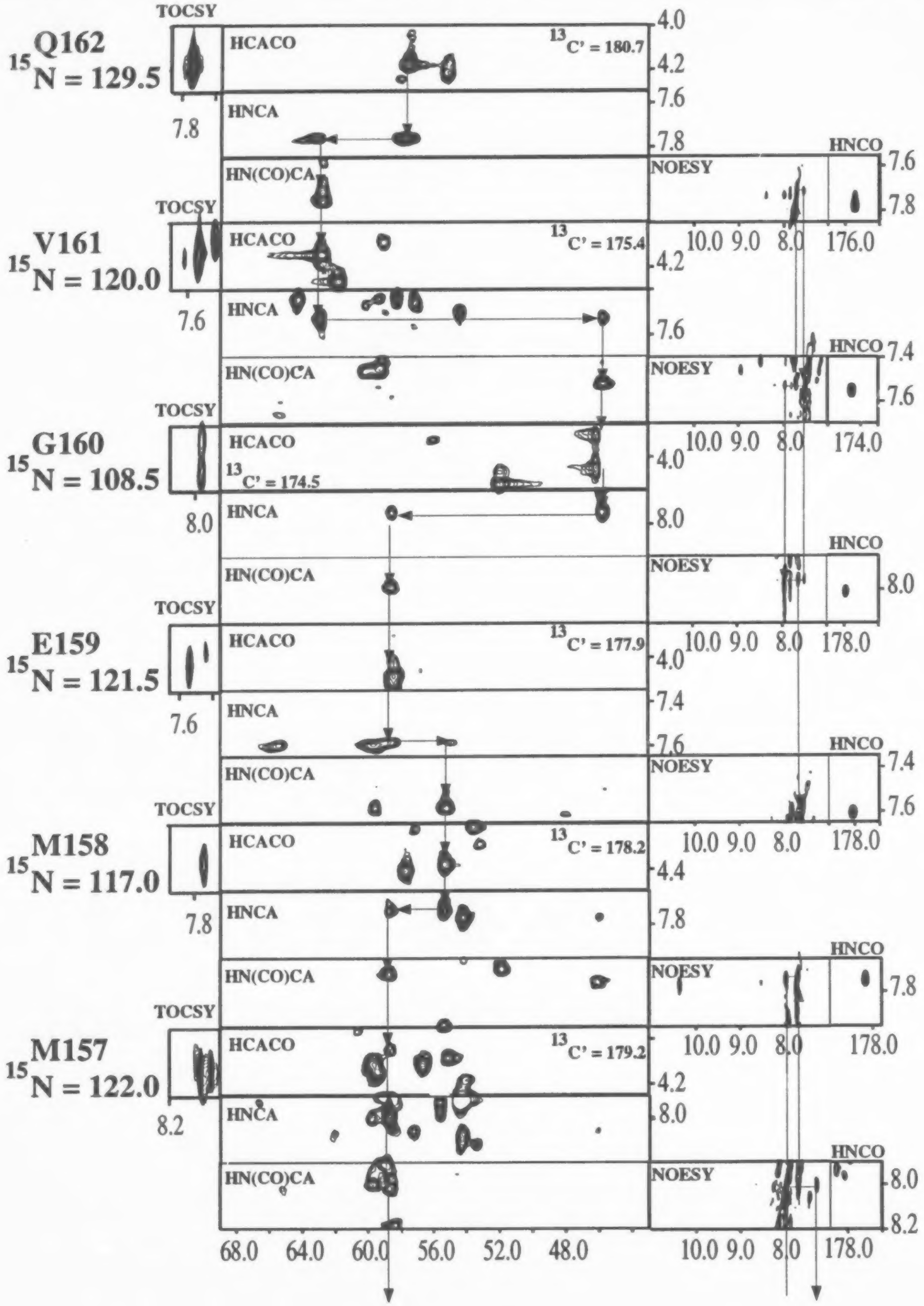
  status(F);                            /* acquisition status */
            setreceiver(t4);
}
```

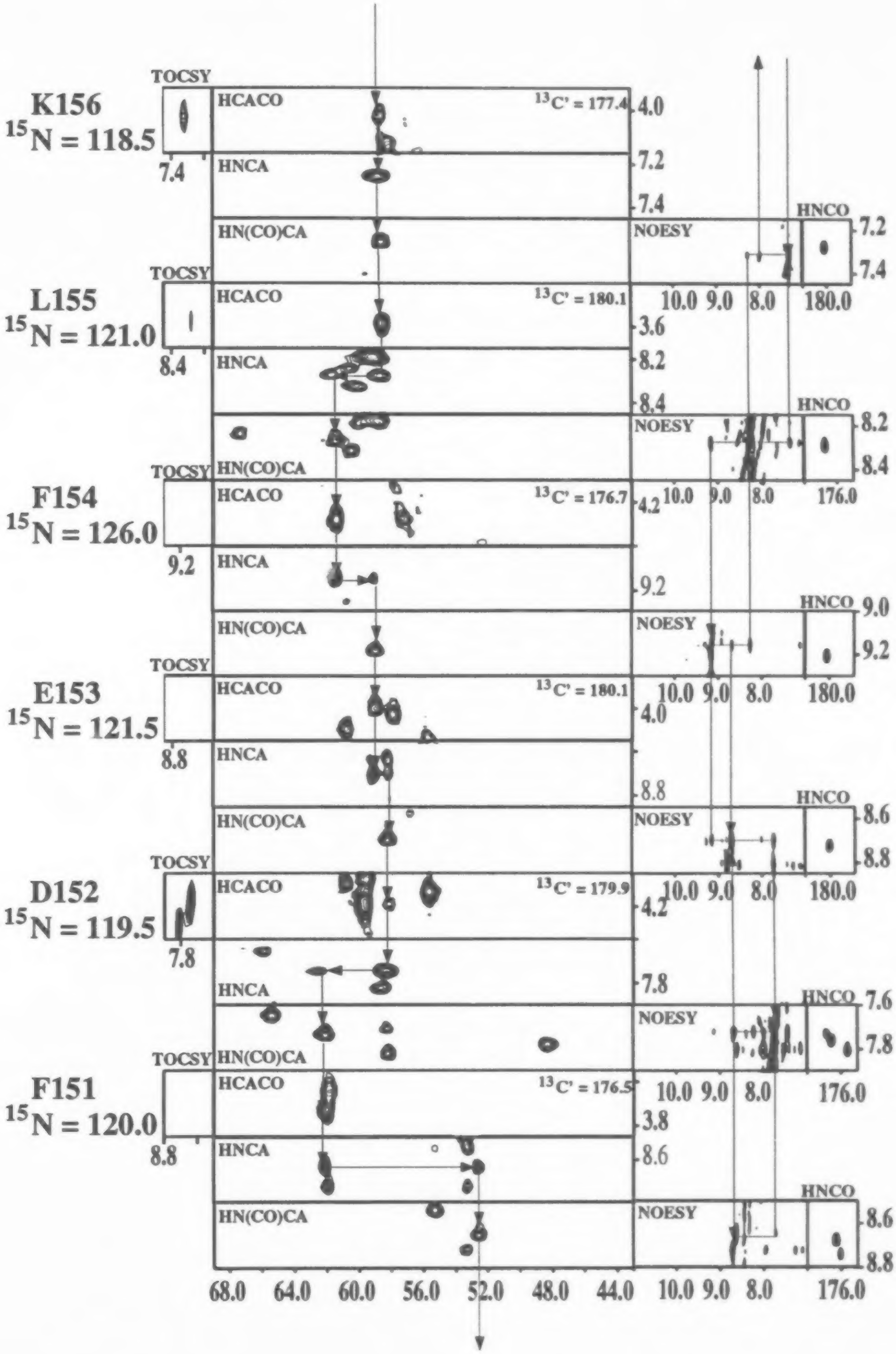
Appendix B:

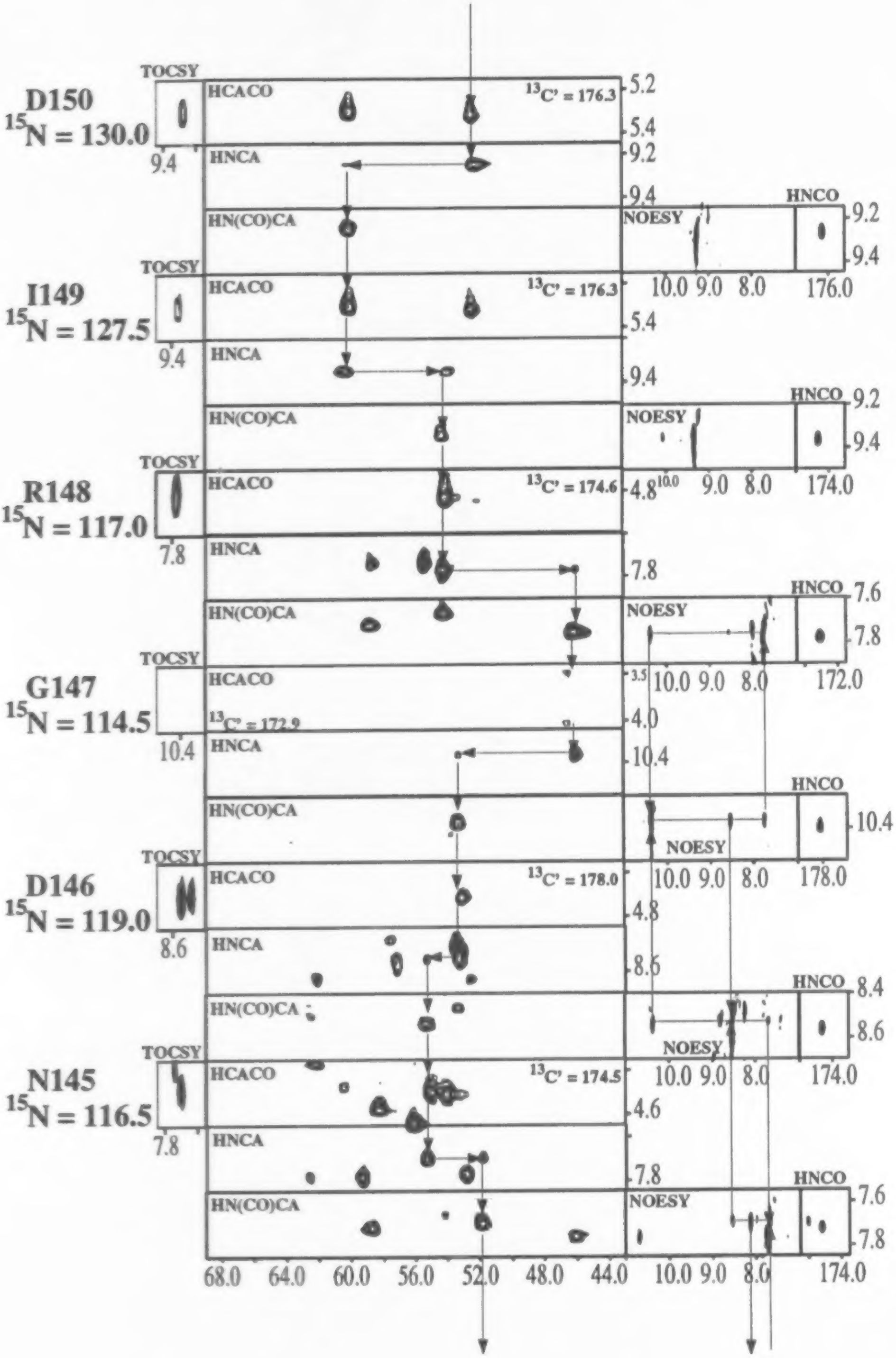
Complete Backbone Assignment of TnC.

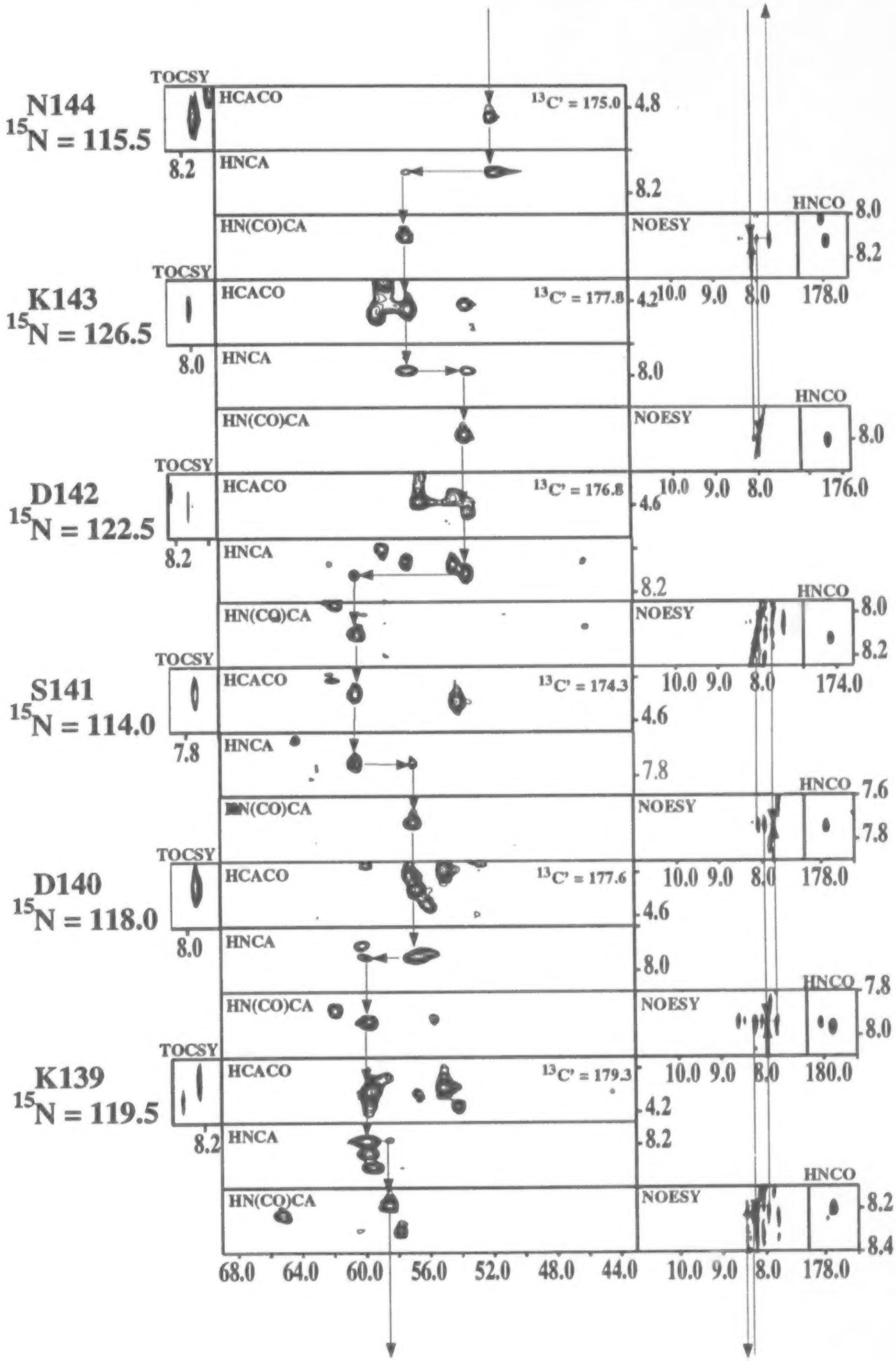
The following figure illustrates the complete backbone assignment of TnC using the following 3D experiments: ^{15}N-edited TOCSY, HCACO, HNCA, HN(CO)CA, ^{15}N-edited NOESY, and HNCO. The residue and the chemical shift corresponding to the ^{15}N plane associated with the ^{15}N-edited TOCSY, HNCA, HN(CO)CA, ^{15}N-edited NOESY, and HNCO are indicated at the left of the figure. For the HCACO, the CO (or C') frequency is indicated on the HCACO slice.

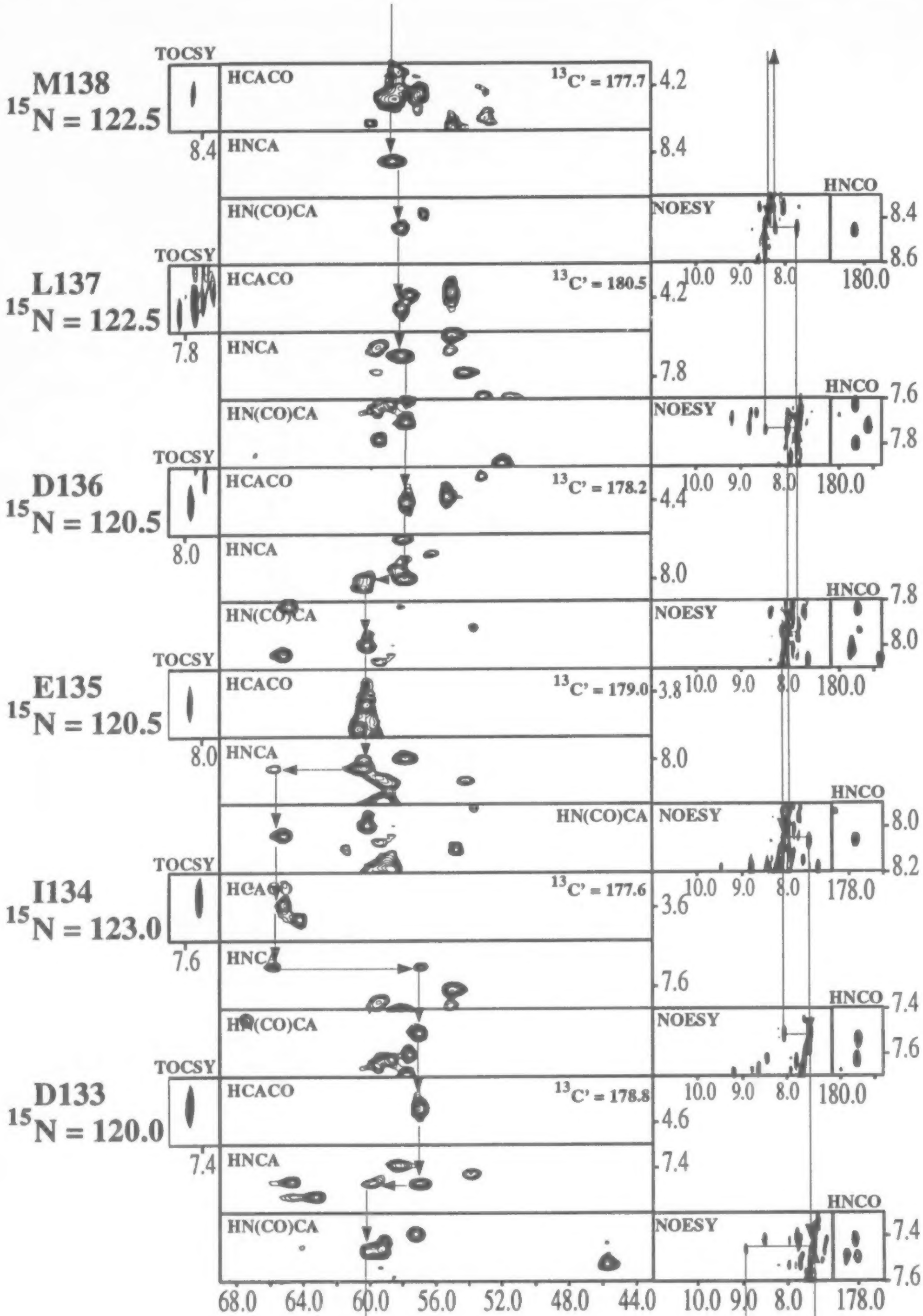
Sequential assignment proceeds starting with the HCACO spectrum where the ^{13}C', ^{13}Cα, and ^{1}Hα resonances are identified. The ^{13}Cα and ^{1}Hα chemical shifts are used to survey the ^{15}N planes of the ^{15}N-edited TOCSY and HNCA spectra to find the ^{15}N and HN chemical shifts for the particular residue. To proceed sequentially, the ^{15}N-edited NOESY experiment is used to find the NH($i\pm1$) chemical shifts together with the HNCA, and the HN(CO)CA to find the ^{13}Cα(i-1) chemical shift, as well as the HNCO experiment to find the ^{13}C'(i-1) chemical shift. The assignment thus starts over again for the next residue, surveying the HCACO, HNCA, and ^{15}N-edited TOCSY experiments first to obtain intraresidue information. A more complete description of the assignment strategy may be found in Chapters III and VI.

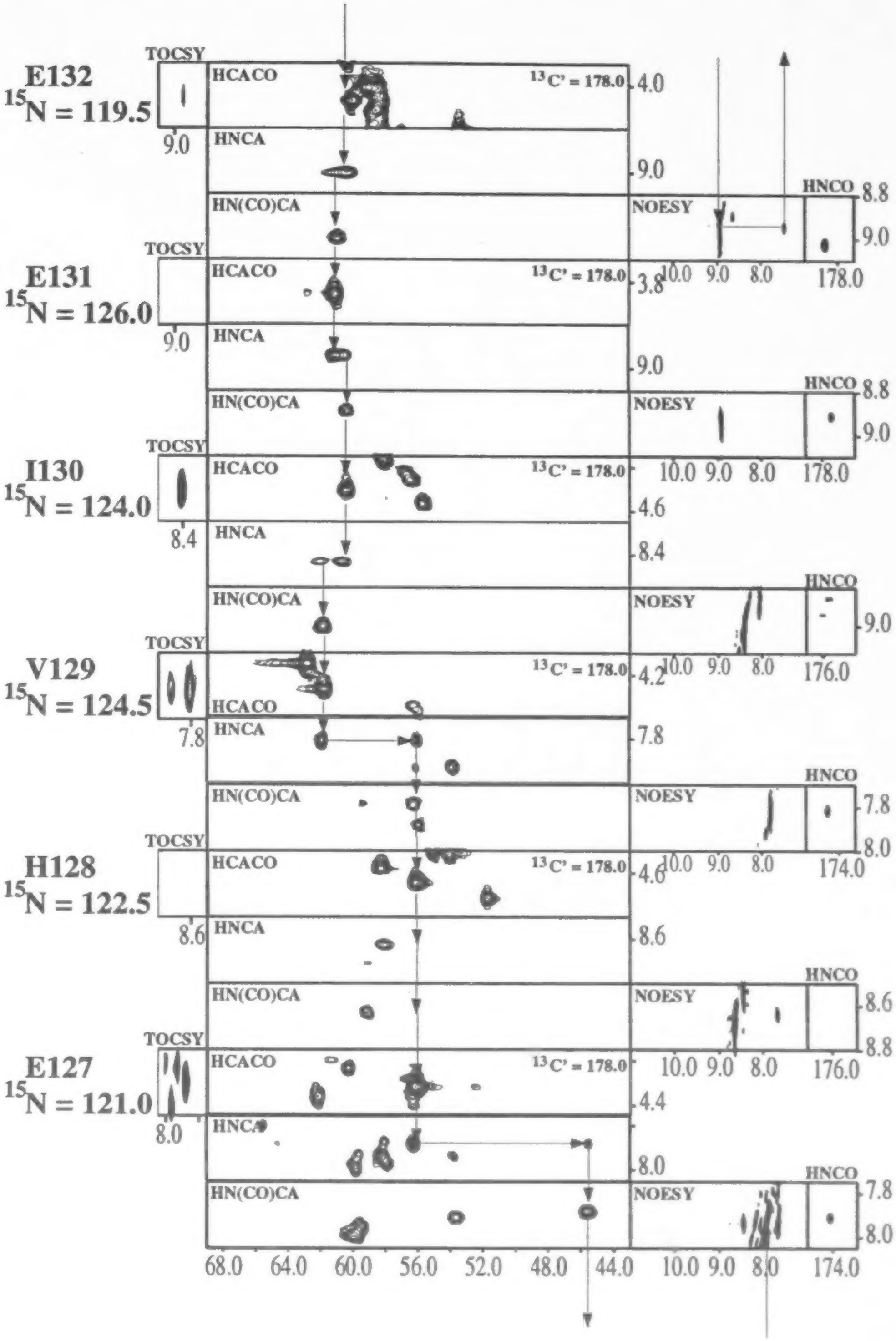

TOCSY
Q162
15N = 129.5
HCACO
13C' = 180.7
HNCA
HN(CO)CA
NOESY
HNCO
V161
15N = 120.0
13C' = 175.4
G160
15N = 108.5
13C' = 174.5
E159
15N = 121.5
13C' = 177.9
M158
15N = 117.0
13C' = 178.2
M157
15N = 122.0
13C' = 179.2
68.0 64.0 60.0 56.0 52.0 48.0
10.0 9.0 8.0
176.0 174.0 178.0
4.0 4.2 7.6 7.8 4.4 8.0 7.4 8.2

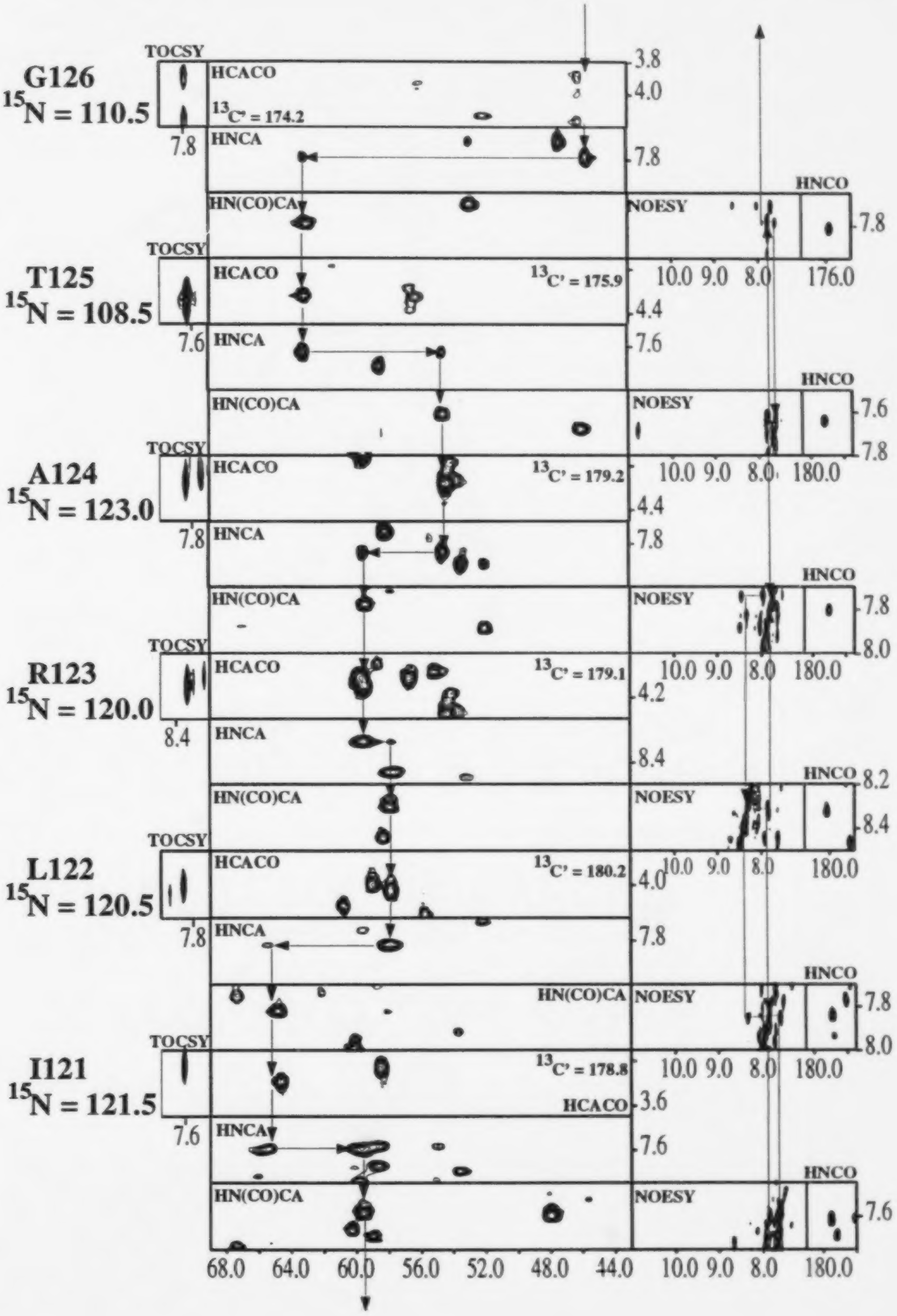

K156
^{15}N = 118.5
L155
^{15}N = 121.0
F154
^{15}N = 126.0
E153
^{15}N = 121.5
D152
^{15}N = 119.5
F151
^{15}N = 120.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 177.4
^{13}C' = 180.1
^{13}C' = 176.7
^{13}C' = 180.1
^{13}C' = 179.9
^{13}C' = 176.5
7.4
8.4
9.2
8.8
7.8
8.8
4.0
7.2
7.4
3.6
8.2
8.4
4.2
9.2
4.0
8.8
4.2
7.8
3.8
8.6
7.2
7.4
8.2
8.4
9.0
9.2
8.6
8.8
7.6
7.8
8.6
8.8
10.0 9.0 8.0
180.0
176.0
68.0
64.0
60.0
56.0
52.0
48.0
44.0

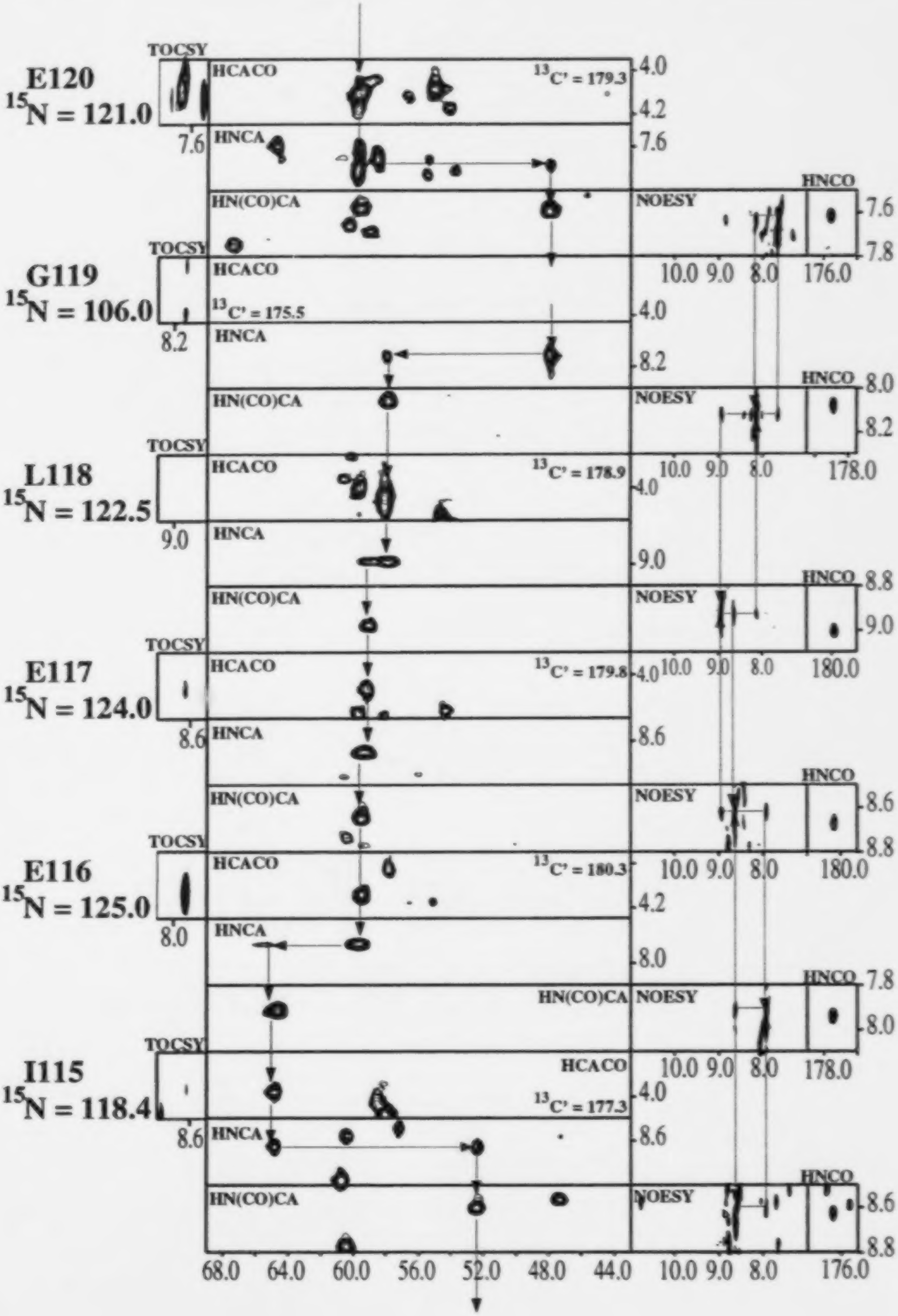

D150
^{15}N = 130.0
I149
^{15}N = 127.5
R148
^{15}N = 117.0
G147
^{15}N = 114.5
D146
^{15}N = 119.0
N145
^{15}N = 116.5
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 176.3
^{13}C' = 176.3
^{13}C' = 174.6
^{13}C' = 172.9
^{13}C' = 178.0
^{13}C' = 174.5
9.4
9.4
7.8
10.4
8.6
7.8
5.2
5.4
9.2
9.4
5.4
9.4
4.8
7.8
3.5
4.0
10.4
4.8
8.6
4.6
7.8
9.2
9.4
9.2
9.4
7.6
7.8
10.4
8.4
8.6
7.6
7.8
10.0 9.0 8.0
176.0
174.0
172.0
178.0
174.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
174.0

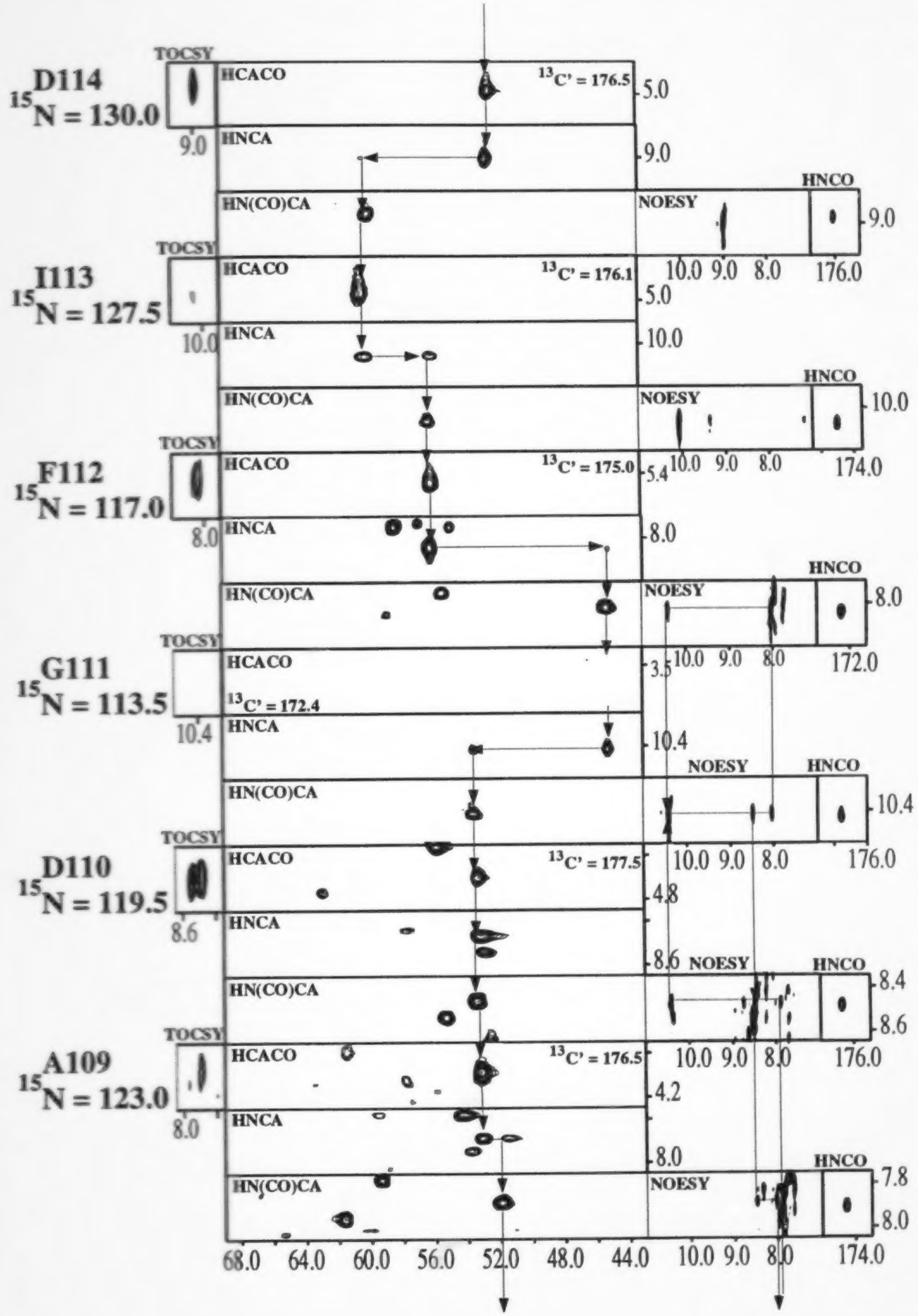

N144
15N = 115.5
TOCSY
8.2
HCACO
13C' = 175.0
4.8
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.0
8.2
K143
15N = 126.5
TOCSY
8.0
HCACO
13C' = 177.8
4.2
10.0 9.0 8.0
178.0
HNCA
8.0
HN(CO)CA
NOESY
HNCO
8.0
D142
15N = 122.5
TOCSY
8.2
HCACO
13C' = 176.8
4.6
10.0 9.0 8.0
176.0
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.0
8.2
S141
15N = 114.0
TOCSY
7.8
HCACO
13C' = 174.3
4.6
10.0 9.0 8.0
174.0
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.6
7.8
D140
15N = 118.0
TOCSY
8.0
HCACO
13C' = 177.6
4.6
10.0 9.0 8.0
178.0
HNCA
8.0
HN(CO)CA
NOESY
HNCO
7.8
8.0
K139
15N = 119.5
TOCSY
8.2
HCACO
13C' = 179.3
4.2
10.0 9.0 8.0
180.0
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.2
8.4
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
178.0

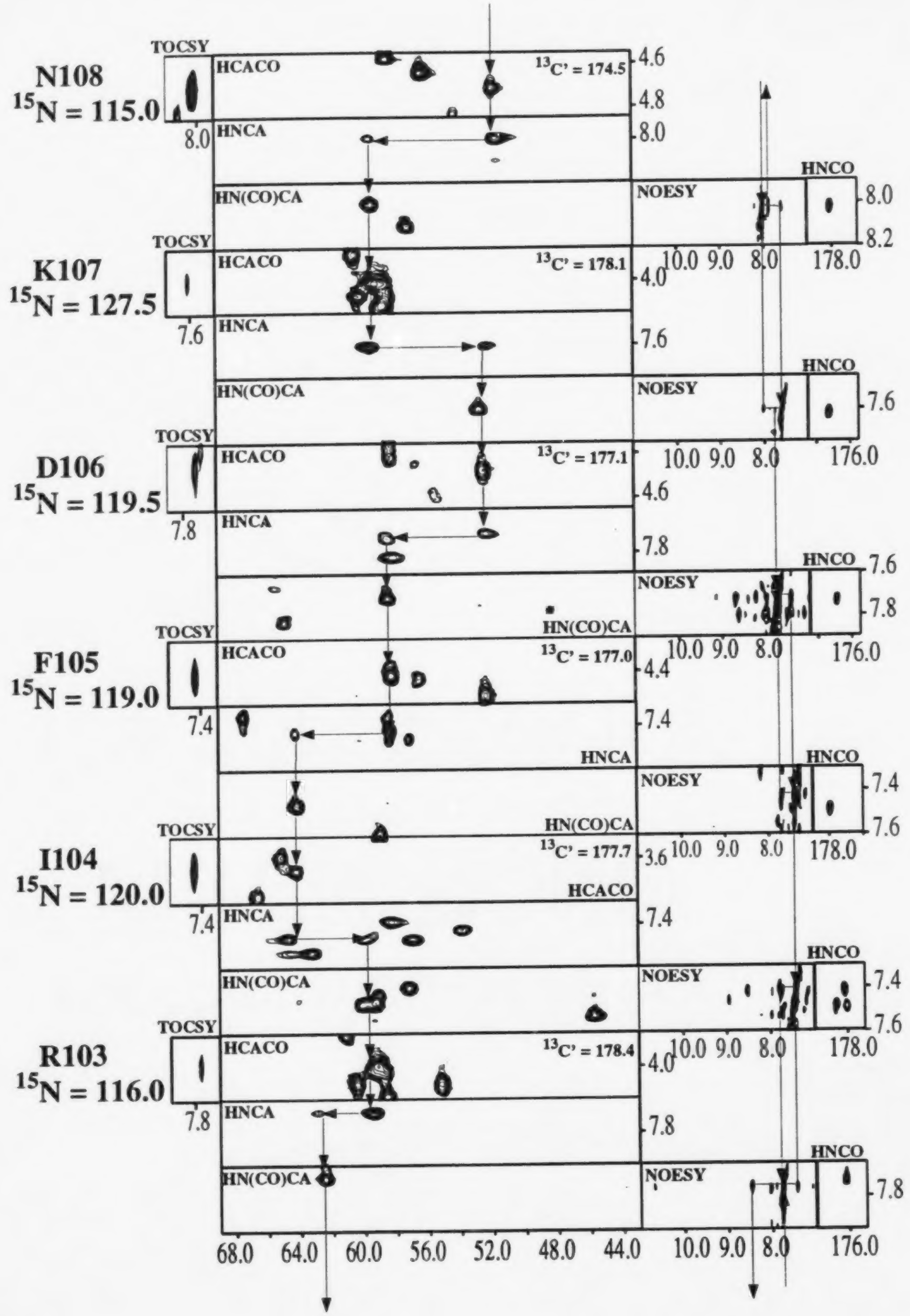

TOCSY
M138
^{15}N = 122.5
HCACO
^{13}C' = 177.7
4.2
8.4
HNCA
8.4
HNCO
HN(CO)CA
NOESY
8.4
8.6
L137
^{15}N = 122.5
HCACO
^{13}C' = 180.5
4.2
10.0 9.0 8.0
180.0
7.8
HNCA
7.8
HNCO
7.6
HN(CO)CA
NOESY
7.8
D136
^{15}N = 120.5
HCACO
^{13}C' = 178.2
4.4
10.0 9.0 8.0
180.0
8.0
HNCA
8.0
HNCO
7.8
HN(CO)CA
NOESY
8.0
E135
^{15}N = 120.5
HCACO
^{13}C' = 179.0
3.8
10.0 9.0 8.0
180.0
8.0
HNCA
8.0
HNCO
HN(CO)CA
NOESY
8.0
8.2
I134
^{15}N = 123.0
HCACO
^{13}C' = 177.6
3.6
10.0 9.0 8.0
178.0
7.6
HNCA
7.6
HNCO
7.4
HN(CO)CA
NOESY
7.6
D133
^{15}N = 120.0
HCACO
^{13}C' = 178.8
4.6
10.0 9.0 8.0
180.0
7.4
HNCA
7.4
HNCO
HN(CO)CA
NOESY
7.4
7.6
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
178.0

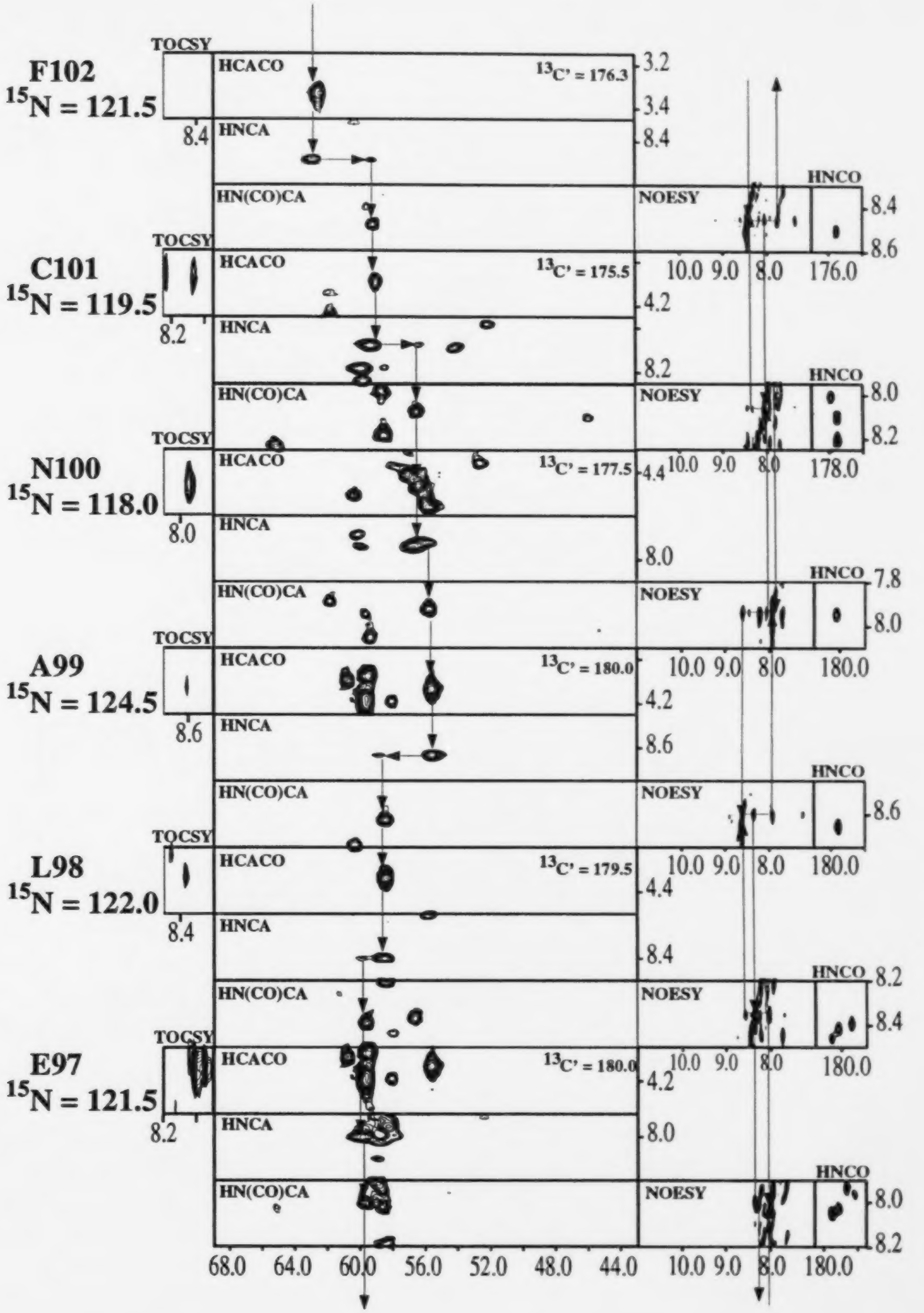

E132
^{15}N = 119.5
E131
^{15}N = 126.0
I130
^{15}N = 124.0
V129
^{15}N = 124.5
H128
^{15}N = 122.5
E127
^{15}N = 121.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 178.0
4.0
9.0
8.8
3.8
4.6
8.4
4.2
7.8
8.6
4.4
8.0
10.0 9.0 8.0
178.0
176.0
174.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0

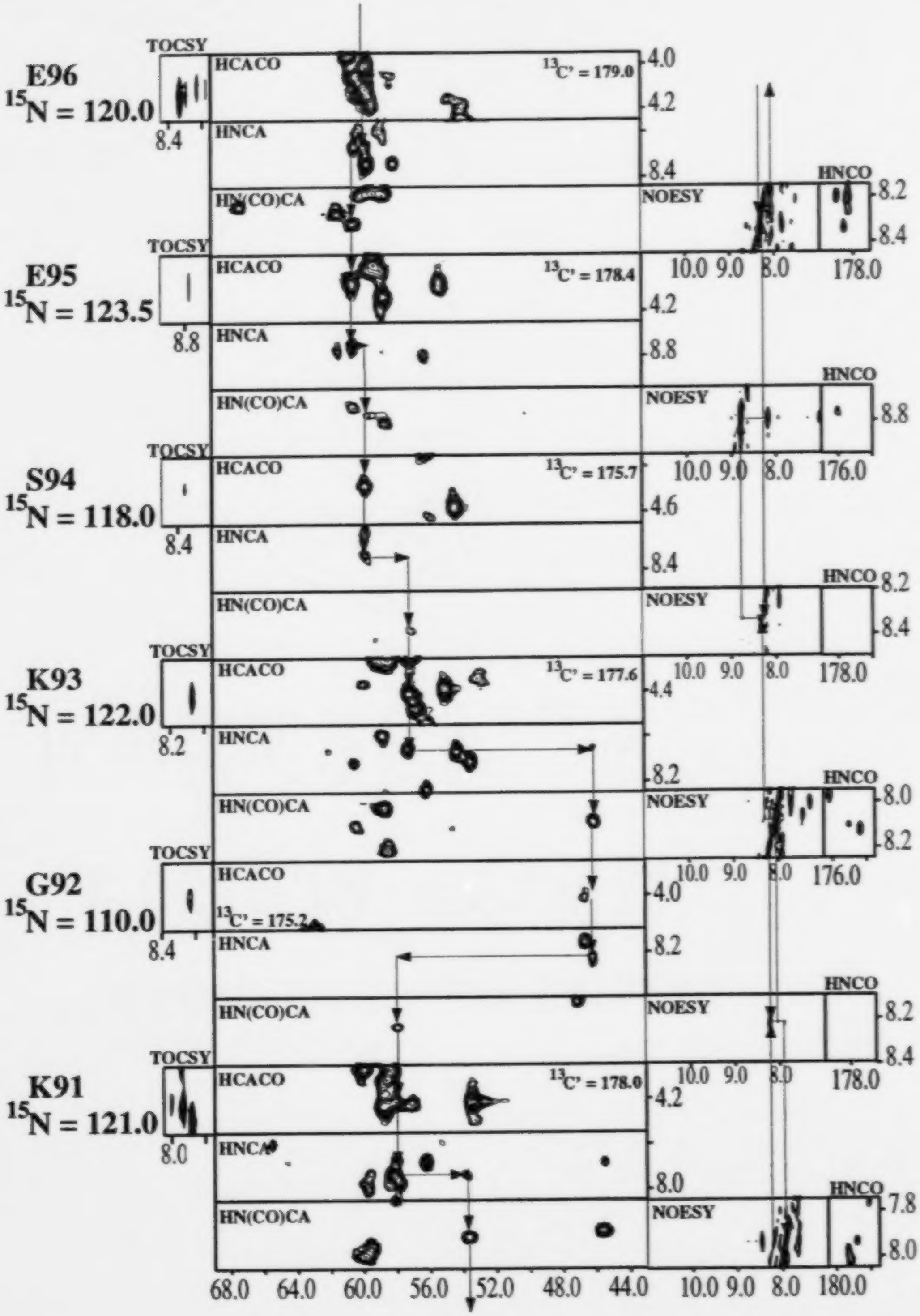

G126
^{15}N = 110.5
TOCSY
HCACO
^{13}C' = 174.2
7.8
HNCA
HN(CO)CA
NOESY
HNCO
3.8
4.0
7.8
7.8
10.0 9.0 8.0
176.0
T125
^{15}N = 108.5
TOCSY
HCACO
^{13}C' = 175.9
4.4
7.6
HNCA
7.6
HN(CO)CA
NOESY
HNCO
7.6
7.8
10.0 9.0 8.0
180.0
A124
^{15}N = 123.0
TOCSY
HCACO
^{13}C' = 179.2
4.4
7.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.8
8.0
10.0 9.0 8.0
180.0
R123
^{15}N = 120.0
TOCSY
HCACO
^{13}C' = 179.1
4.2
8.4
HNCA
8.4
HN(CO)CA
NOESY
HNCO
8.2
8.4
10.0 9.0 8.0
180.0
L122
^{15}N = 120.5
TOCSY
HCACO
^{13}C' = 180.2
4.0
7.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.8
8.0
10.0 9.0 8.0
180.0
I121
^{15}N = 121.5
TOCSY
^{13}C' = 178.8
HCACO
3.6
7.6
HNCA
7.6
HN(CO)CA
NOESY
HNCO
7.6
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
180.0

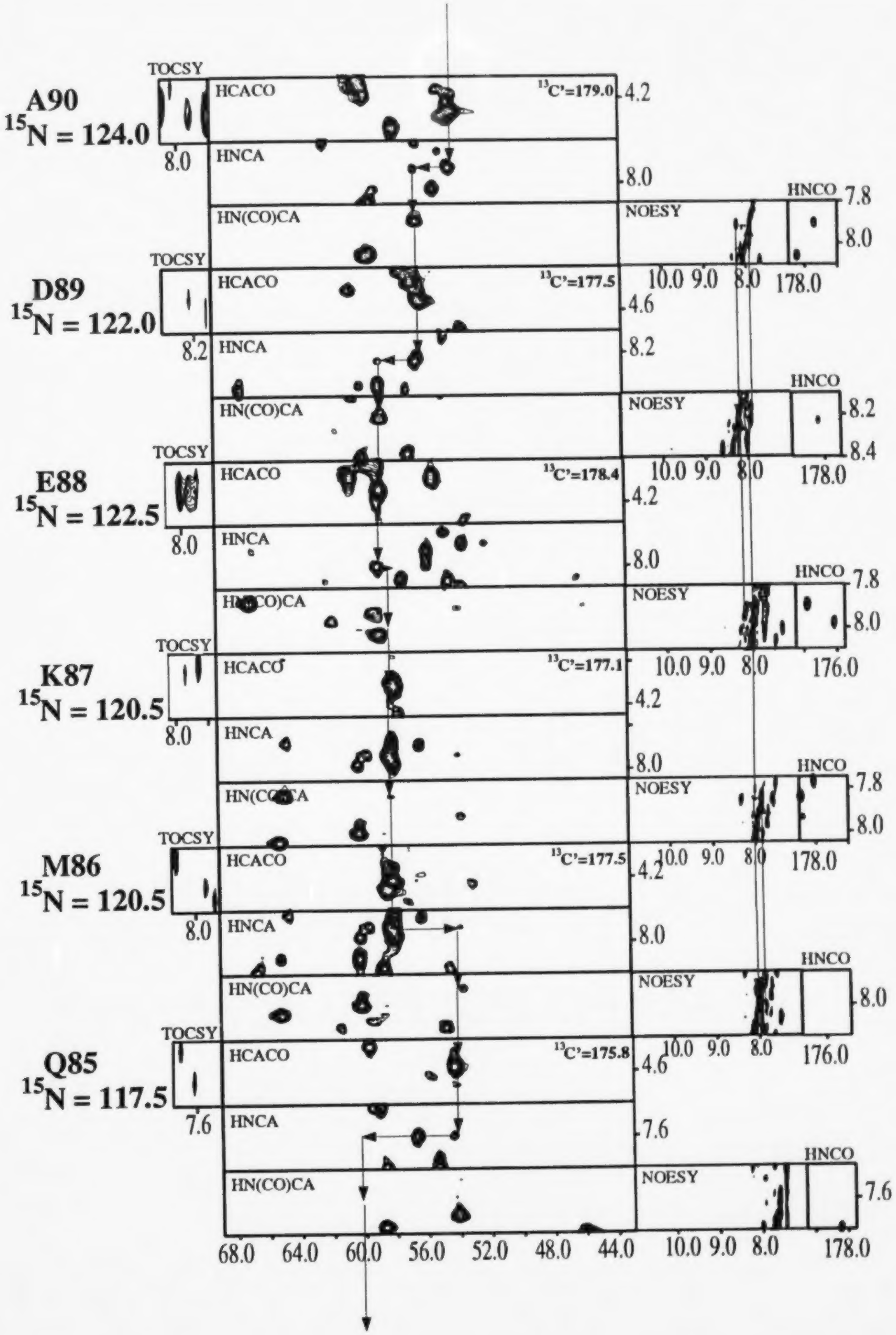

E120
^{15}N = 121.0
G119
^{15}N = 106.0
L118
^{15}N = 122.5
E117
^{15}N = 124.0
E116
^{15}N = 125.0
I115
^{15}N = 118.4
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 179.3
^{13}C' = 175.5
^{13}C' = 178.9
^{13}C' = 179.8
^{13}C' = 180.3
^{13}C' = 177.3
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
176.0
178.0
180.0
4.0
4.2
7.6
7.8
8.0
8.2
8.6
8.8
9.0

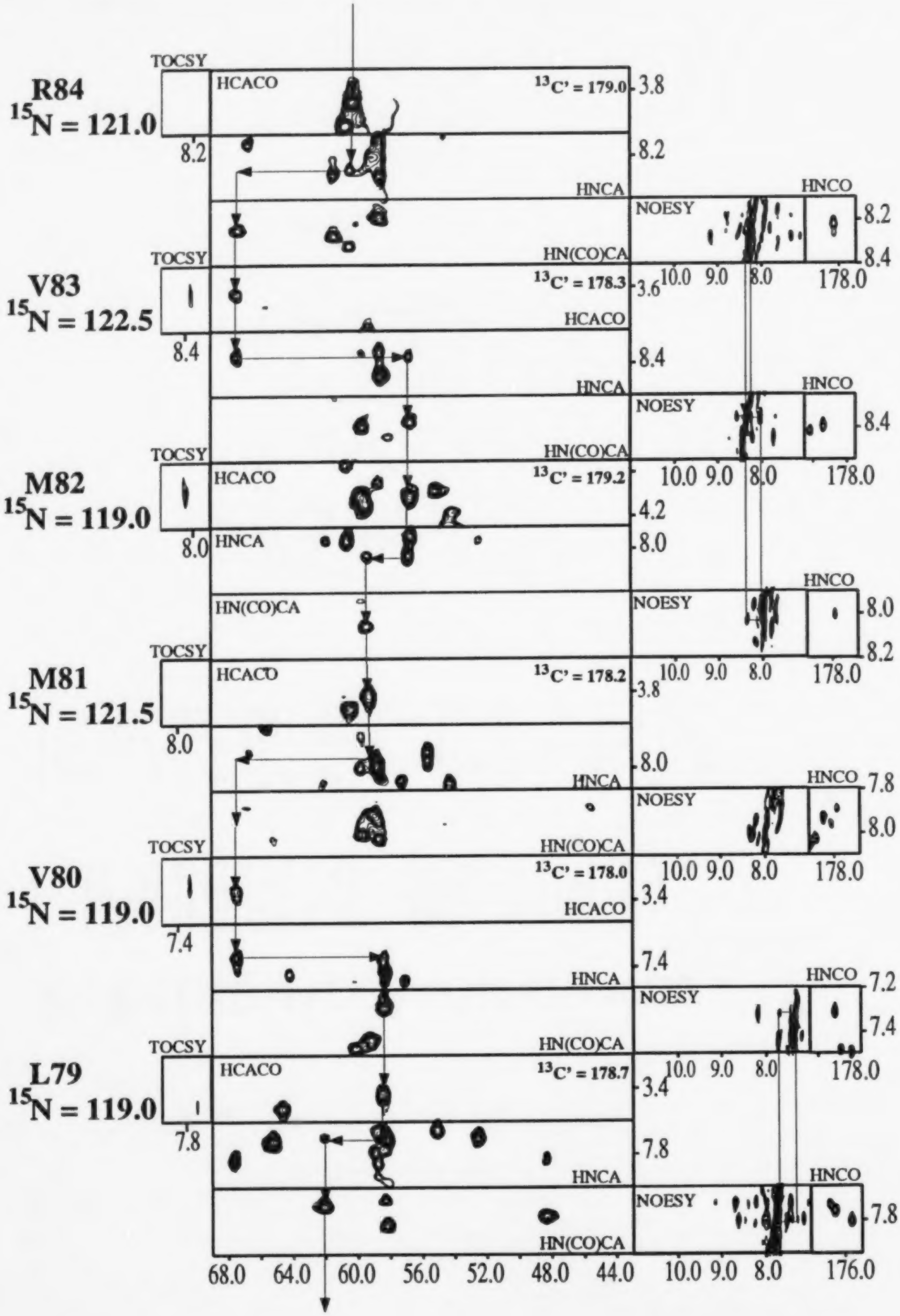

D114
^{15}N = 130.0
TOCSY
9.0
HCACO
^{13}C' = 176.5
5.0
HNCA
9.0
HN(CO)CA
NOESY
HNCO
9.0
10.0 9.0 8.0
176.0
I113
^{15}N = 127.5
TOCSY
10.0
HCACO
^{13}C' = 176.1
5.0
HNCA
10.0
HN(CO)CA
NOESY
HNCO
10.0
10.0 9.0 8.0
174.0
F112
^{15}N = 117.0
TOCSY
8.0
HCACO
^{13}C' = 175.0
5.4
HNCA
8.0
HN(CO)CA
NOESY
HNCO
8.0
10.0 9.0 8.0
172.0
G111
^{15}N = 113.5
TOCSY
10.4
HCACO
^{13}C' = 172.4
3.5
HNCA
10.4
HN(CO)CA
NOESY
HNCO
10.4
10.0 9.0 8.0
176.0
D110
^{15}N = 119.5
TOCSY
8.6
HCACO
^{13}C' = 177.5
4.8
HNCA
8.6
HN(CO)CA
NOESY
HNCO
8.4
8.6
10.0 9.0 8.0
176.0
A109
^{15}N = 123.0
TOCSY
8.0
HCACO
^{13}C' = 176.5
4.2
HNCA
8.0
HN(CO)CA
NOESY
HNCO
7.8
8.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
174.0

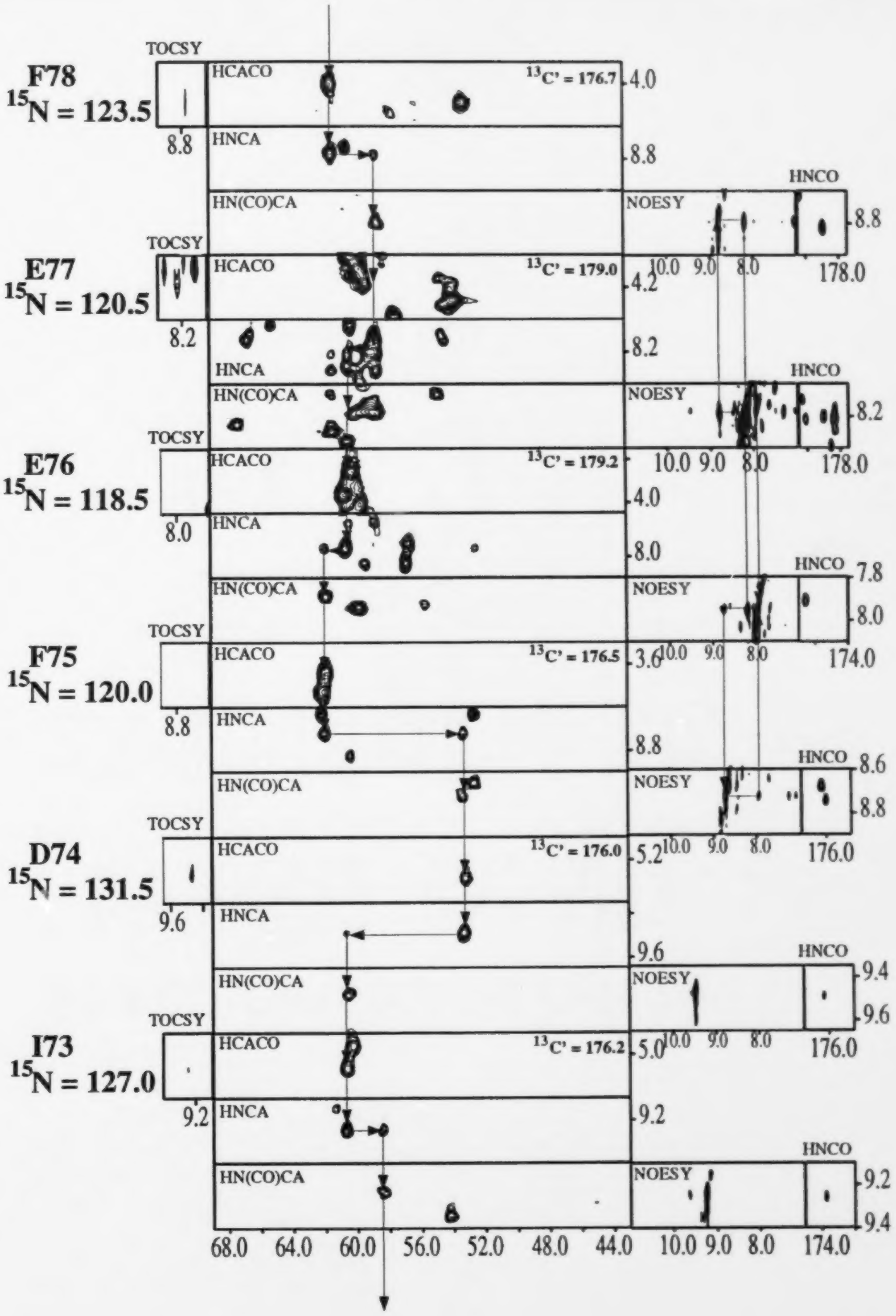

N108
^{15}N = 115.0
K107
^{15}N = 127.5
D106
^{15}N = 119.5
F105
^{15}N = 119.0
I104
^{15}N = 120.0
R103
^{15}N = 116.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 174.5
^{13}C' = 178.1
^{13}C' = 177.1
^{13}C' = 177.0
^{13}C' = 177.7
^{13}C' = 178.4
68.0
64.0
60.0
56.0
52.0
48.0
44.0
10.0
9.0
8.0
178.0
176.0
4.6
4.8
8.0
8.2
4.0
7.6
4.4
7.4
3.6
7.8

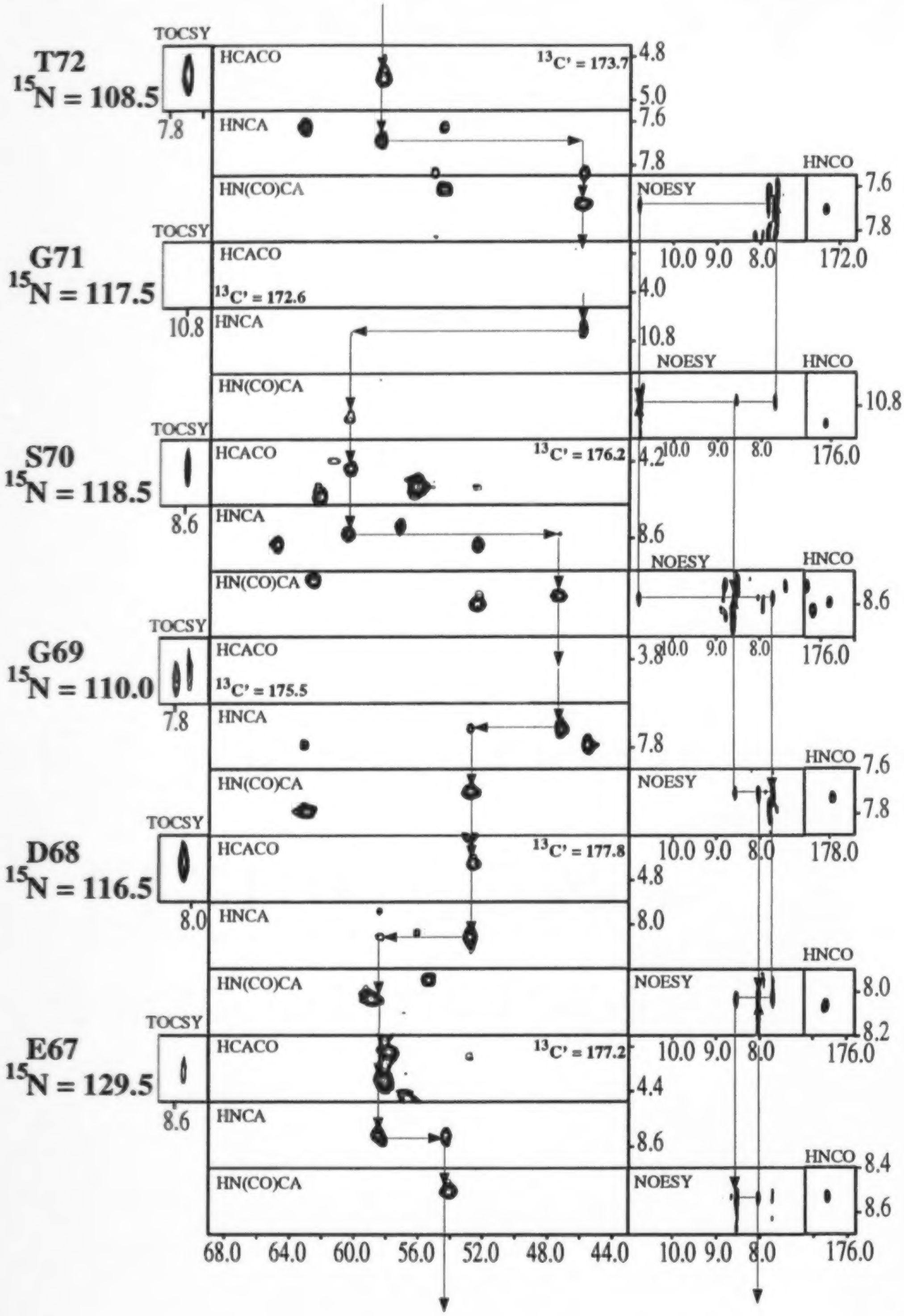

F102
^{15}N = 121.5
C101
^{15}N = 119.5
N100
^{15}N = 118.0
A99
^{15}N = 124.5
L98
^{15}N = 122.0
E97
^{15}N = 121.5
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 176.3
^{13}C' = 175.5
^{13}C' = 177.5
^{13}C' = 180.0
^{13}C' = 179.5
^{13}C' = 180.0
68.0
64.0
60.0
56.0
52.0
48.0
44.0
10.0
9.0
8.0
176.0
178.0
180.0
3.2
3.4
4.2
4.4
7.8
8.0
8.2
8.4
8.6

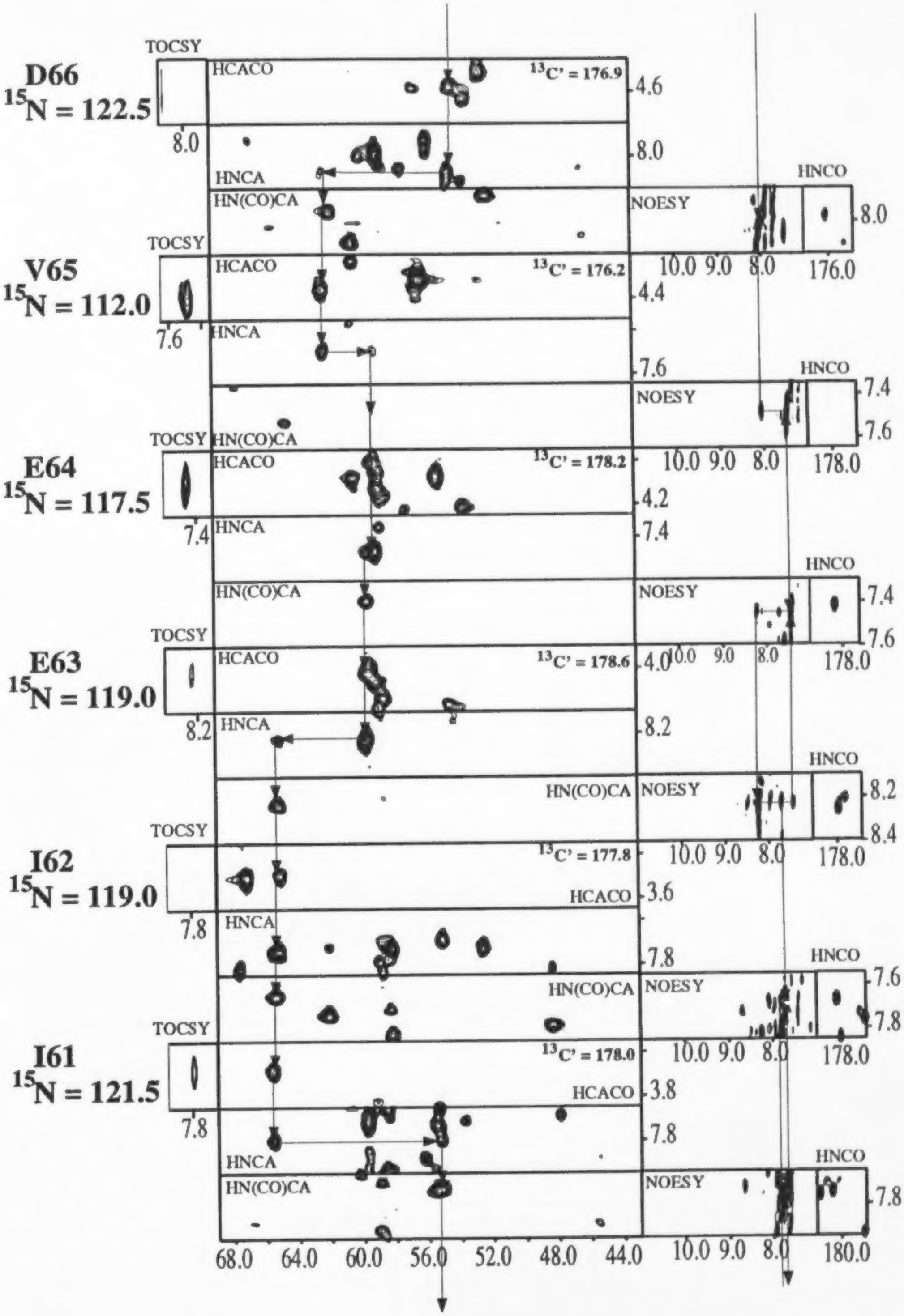

E96
^{15}N = 120.0
TOCSY
HCACO
^{13}C' = 179.0
HNCA
HN(CO)CA
NOESY
HNCO
E95
^{15}N = 123.5
^{13}C' = 178.4
S94
^{15}N = 118.0
^{13}C' = 175.7
K93
^{15}N = 122.0
^{13}C' = 177.6
G92
^{15}N = 110.0
^{13}C' = 175.2
K91
^{15}N = 121.0
^{13}C' = 178.0
4.0
4.2
8.4
8.2
8.8
4.6
4.4
7.8
8.0
10.0
9.0
178.0
176.0
180.0
68.0
64.0
60.0
56.0
52.0
48.0
44.0

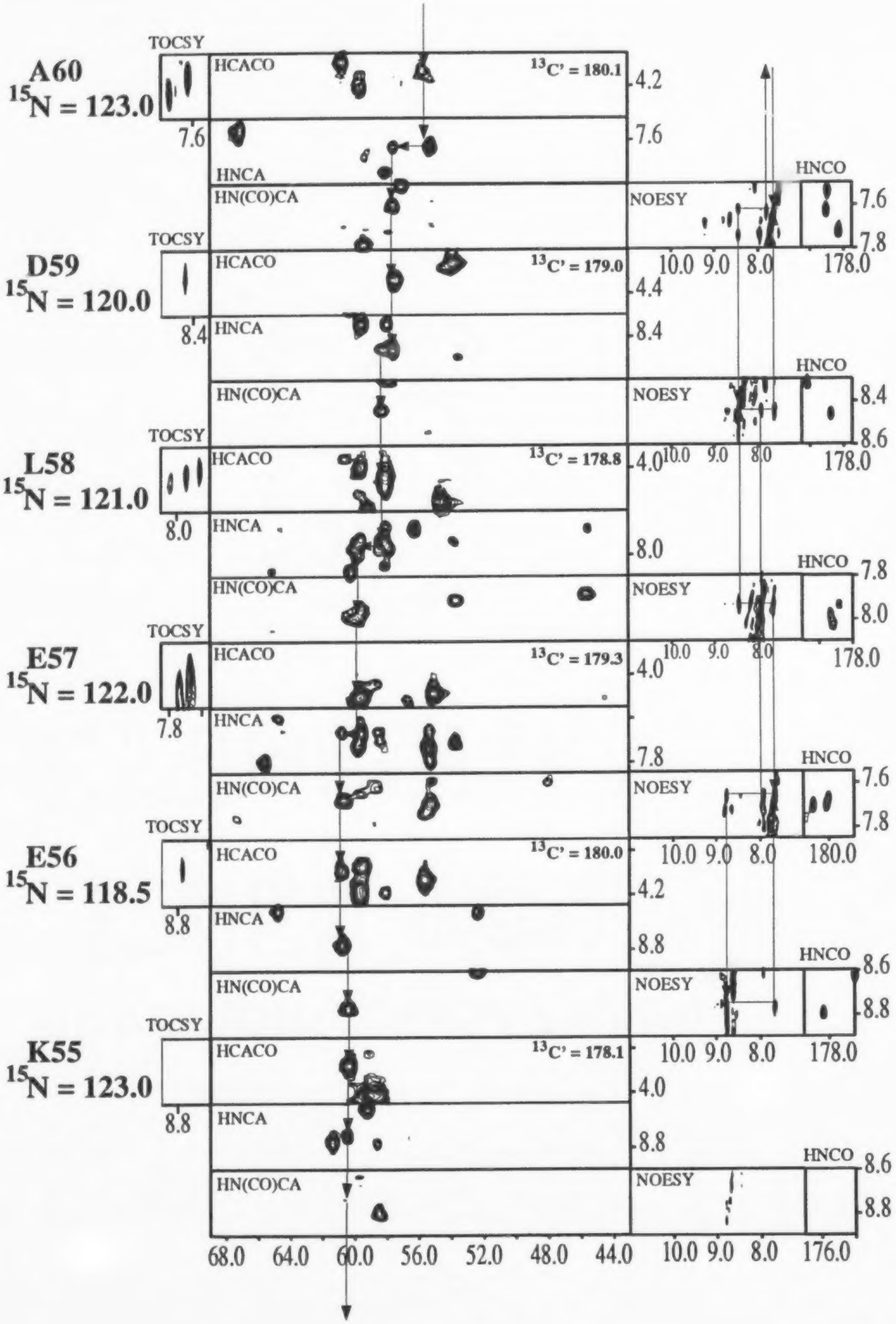

A90
^{15}N = 124.0
D89
^{15}N = 122.0
E88
^{15}N = 122.5
K87
^{15}N = 120.5
M86
^{15}N = 120.5
Q85
^{15}N = 117.5
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C'=179.0
^{13}C'=177.5
^{13}C'=178.4
^{13}C'=177.1
^{13}C'=177.5
^{13}C'=175.8
4.2
4.6
7.6
7.8
8.0
8.2
8.4
10.0
9.0
176.0
178.0
68.0
64.0
60.0
56.0
52.0
48.0
44.0

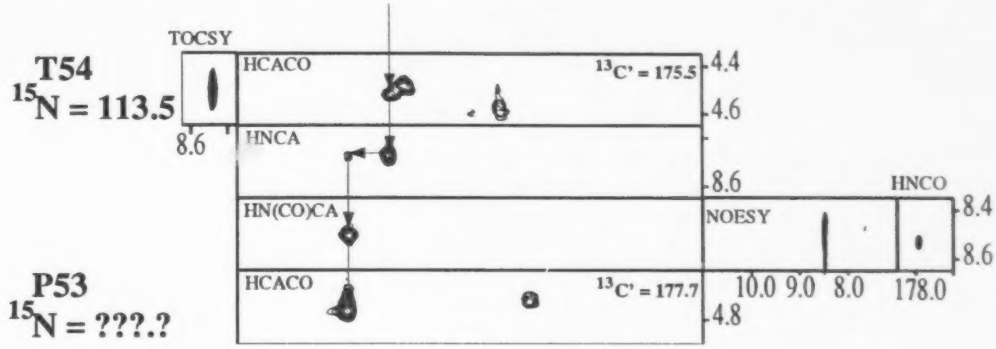

R84
^{15}N = 121.0
V83
^{15}N = 122.5
M82
^{15}N = 119.0
M81
^{15}N = 121.5
V80
^{15}N = 119.0
L79
^{15}N = 119.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 179.0
^{13}C' = 178.3
^{13}C' = 179.2
^{13}C' = 178.2
^{13}C' = 178.0
^{13}C' = 178.7
68.0
64.0
60.0
56.0
52.0
48.0
44.0
10.0
9.0
8.0
178.0
176.0
3.8
8.2
8.4
3.6
4.2
8.0
7.8
3.4
7.4
7.2

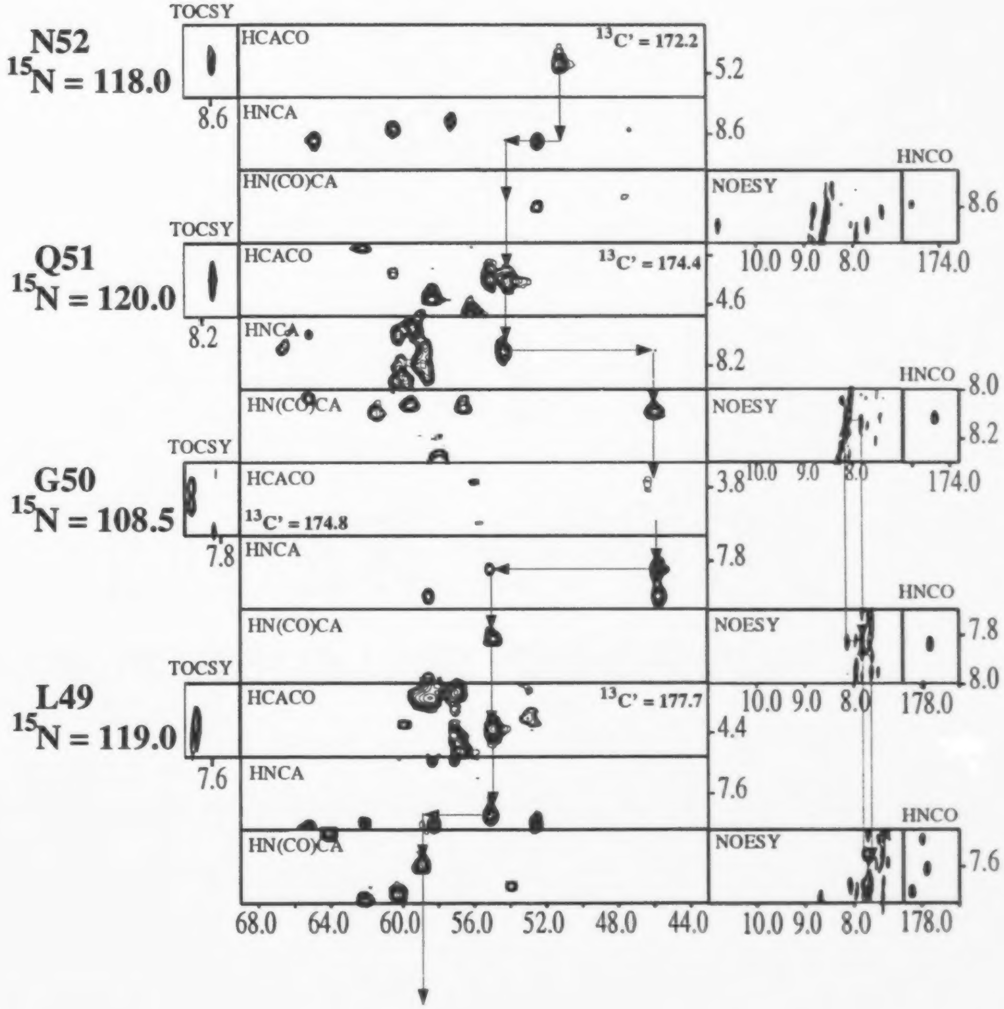

F78
15N = 123.5
E77
15N = 120.5
E76
15N = 118.5
F75
15N = 120.0
D74
15N = 131.5
I73
15N = 127.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
13C' = 176.7
13C' = 179.0
13C' = 179.2
13C' = 176.5
13C' = 176.0
13C' = 176.2
8.8
8.2
8.0
9.6
9.2
4.0
4.2
3.6
5.2
5.0
7.8
8.6
9.4
178.0
174.0
176.0
68.0
64.0
60.0
56.0
52.0
48.0
44.0
10.0
9.0
8.0

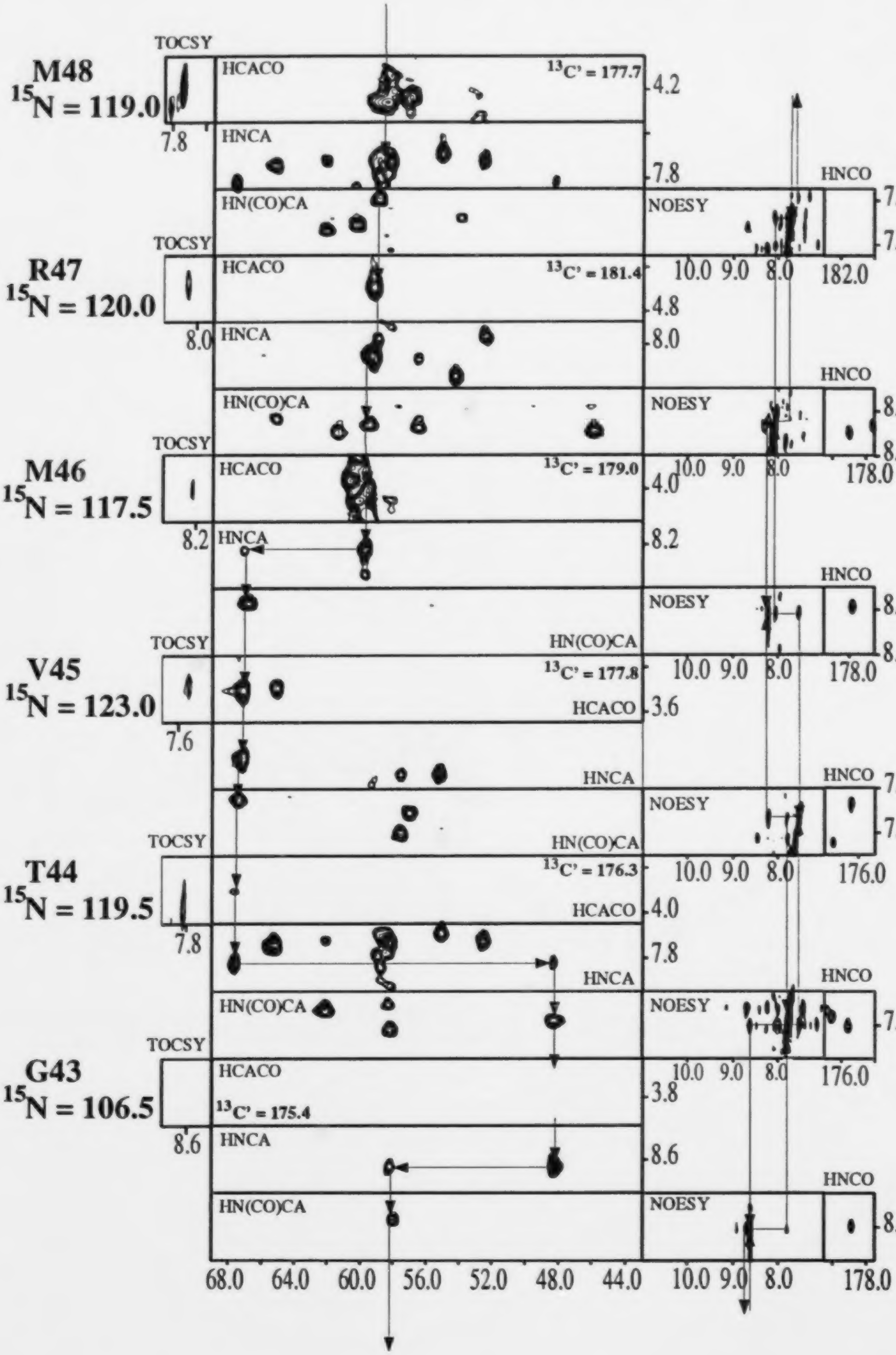

TOCSY
T72
^{15}N = 108.5
7.8
HCACO
^{13}C' = 173.7
4.8
5.0
HNCA
7.6
7.8
HN(CO)CA
NOESY
HNCO
7.6
7.8
10.0 9.0 8.0
172.0
TOCSY
G71
^{15}N = 117.5
HCACO
^{13}C' = 172.6
4.0
10.8
HNCA
10.8
NOESY
HNCO
HN(CO)CA
10.8
10.0 9.0 8.0
176.0
TOCSY
S70
^{15}N = 118.5
HCACO
^{13}C' = 176.2
4.2
8.6
HNCA
8.6
NOESY
HNCO
HN(CO)CA
8.6
10.0 9.0 8.0
176.0
TOCSY
G69
^{15}N = 110.0
HCACO
^{13}C' = 175.5
3.8
7.8
HNCA
7.8
HNCO
7.6
HN(CO)CA
NOESY
7.8
10.0 9.0 8.0
178.0
TOCSY
D68
^{15}N = 116.5
HCACO
^{13}C' = 177.8
4.8
8.0
HNCA
8.0
HNCO
HN(CO)CA
NOESY
8.0
8.2
10.0 9.0 8.0
176.0
TOCSY
E67
^{15}N = 129.5
HCACO
^{13}C' = 177.2
4.4
8.6
HNCA
8.6
HNCO
8.4
HN(CO)CA
NOESY
8.6
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
176.0

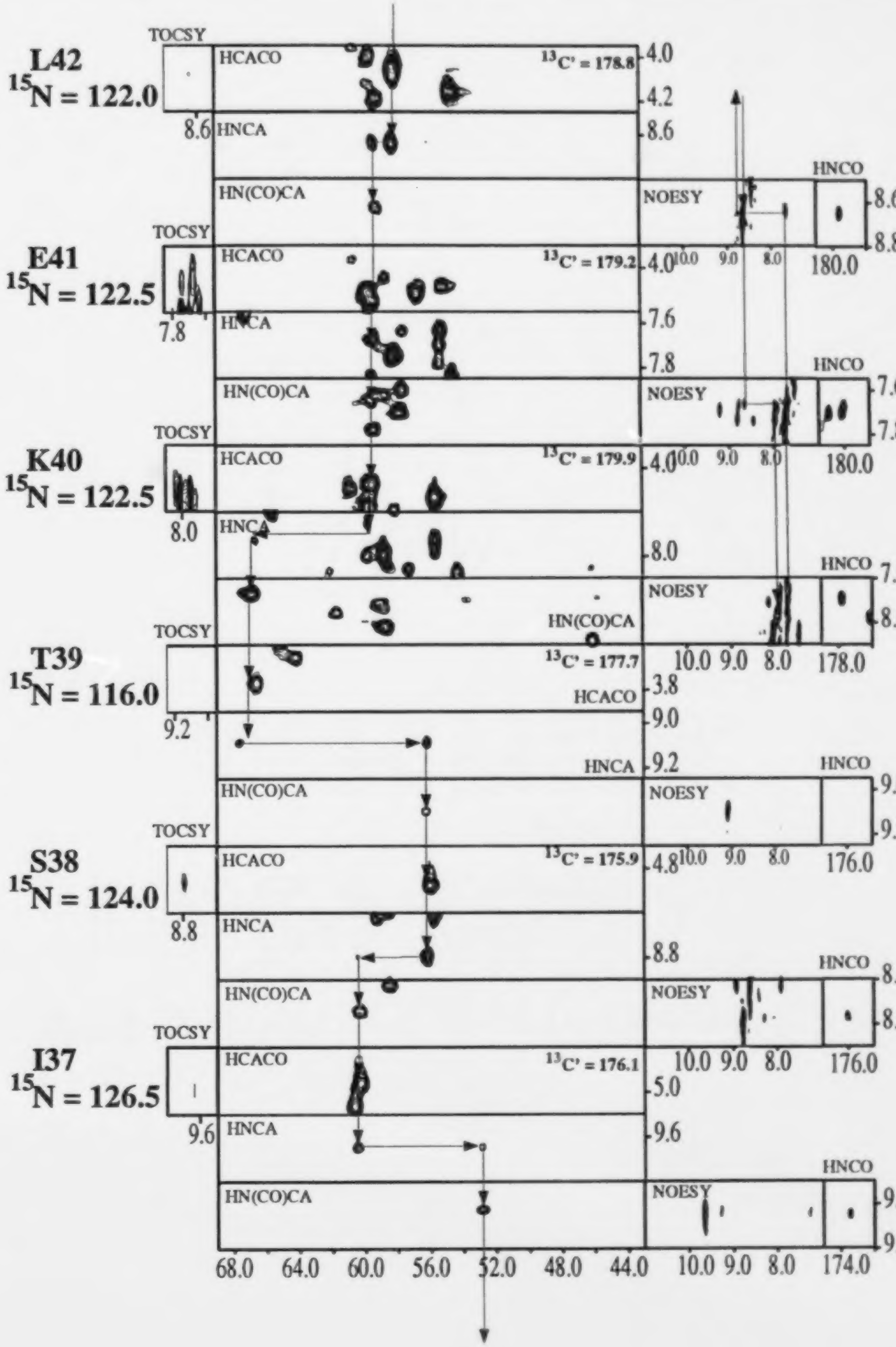

D66
$^{15}N = 122.5$
V65
$^{15}N = 112.0$
E64
$^{15}N = 117.5$
E63
$^{15}N = 119.0$
I62
$^{15}N = 119.0$
I61
$^{15}N = 121.5$
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
$^{13}C' = 176.9$
$^{13}C' = 176.2$
$^{13}C' = 178.2$
$^{13}C' = 178.6$
$^{13}C' = 177.8$
$^{13}C' = 178.0$
8.0
7.6
7.4
8.2
7.8
4.6
4.4
4.2
4.0
3.6
3.8
10.0 9.0 8.0
176.0
178.0
180.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0

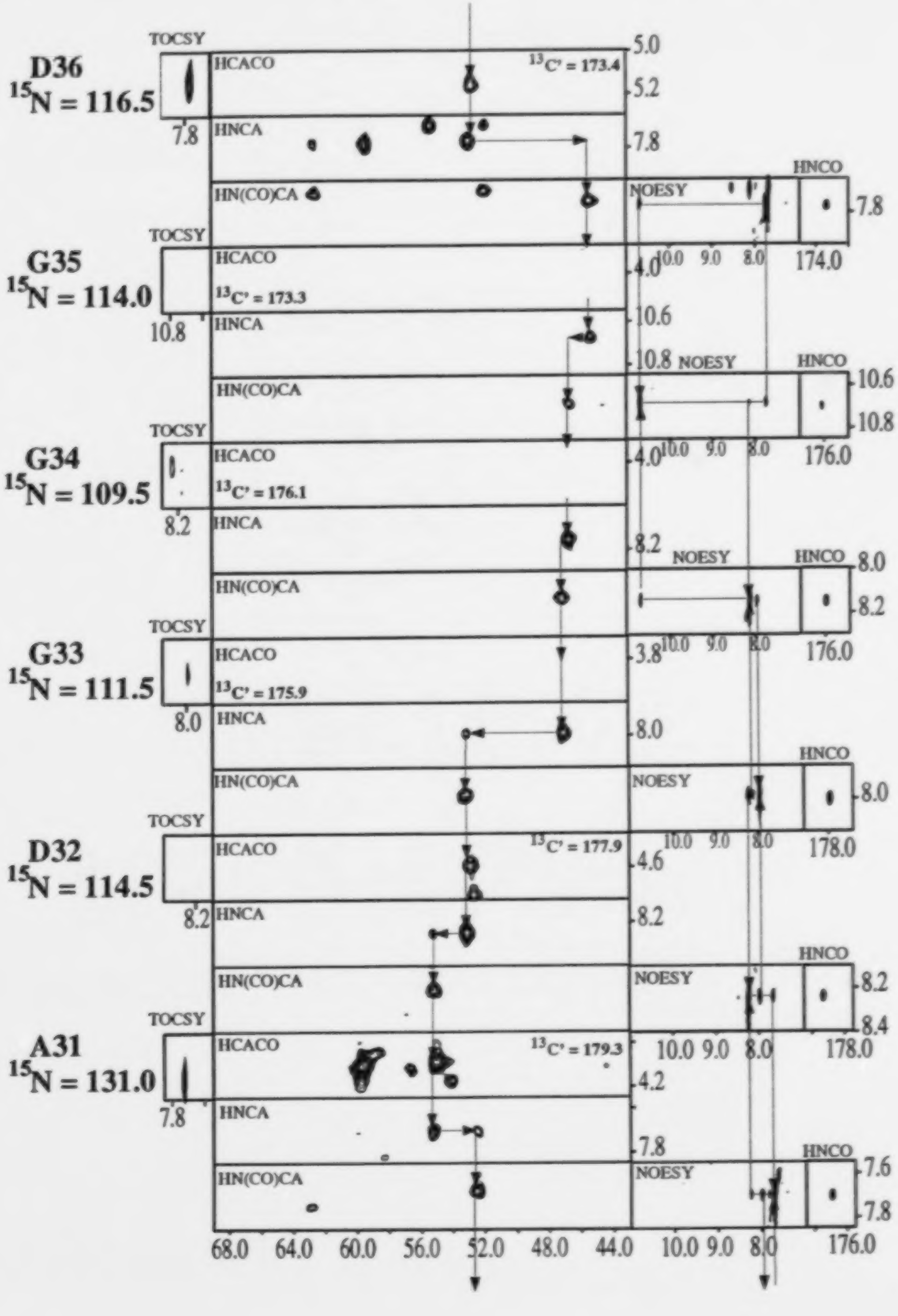

A60
^{15}N = 123.0
TOCSY
HCACO
^{13}C' = 180.1
4.2
7.6
HNCA
7.6
HN(CO)CA
NOESY
HNCO
7.6
7.8
10.0 9.0 8.0
178.0
D59
^{15}N = 120.0
TOCSY
HCACO
^{13}C' = 179.0
4.4
8.4
HNCA
8.4
HN(CO)CA
NOESY
HNCO
8.4
8.6
10.0 9.0 8.0
178.0
L58
^{15}N = 121.0
TOCSY
HCACO
^{13}C' = 178.8
4.0
8.0
HNCA
8.0
HN(CO)CA
NOESY
HNCO
7.8
8.0
10.0 9.0 8.0
178.0
E57
^{15}N = 122.0
TOCSY
HCACO
^{13}C' = 179.3
4.0
7.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.6
7.8
10.0 9.0 8.0
180.0
E56
^{15}N = 118.5
TOCSY
HCACO
^{13}C' = 180.0
4.2
8.8
HNCA
8.8
HN(CO)CA
NOESY
HNCO
8.6
8.8
10.0 9.0 8.0
178.0
K55
^{15}N = 123.0
TOCSY
HCACO
^{13}C' = 178.1
4.0
8.8
HNCA
8.8
HN(CO)CA
NOESY
HNCO
8.6
8.8
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
176.0

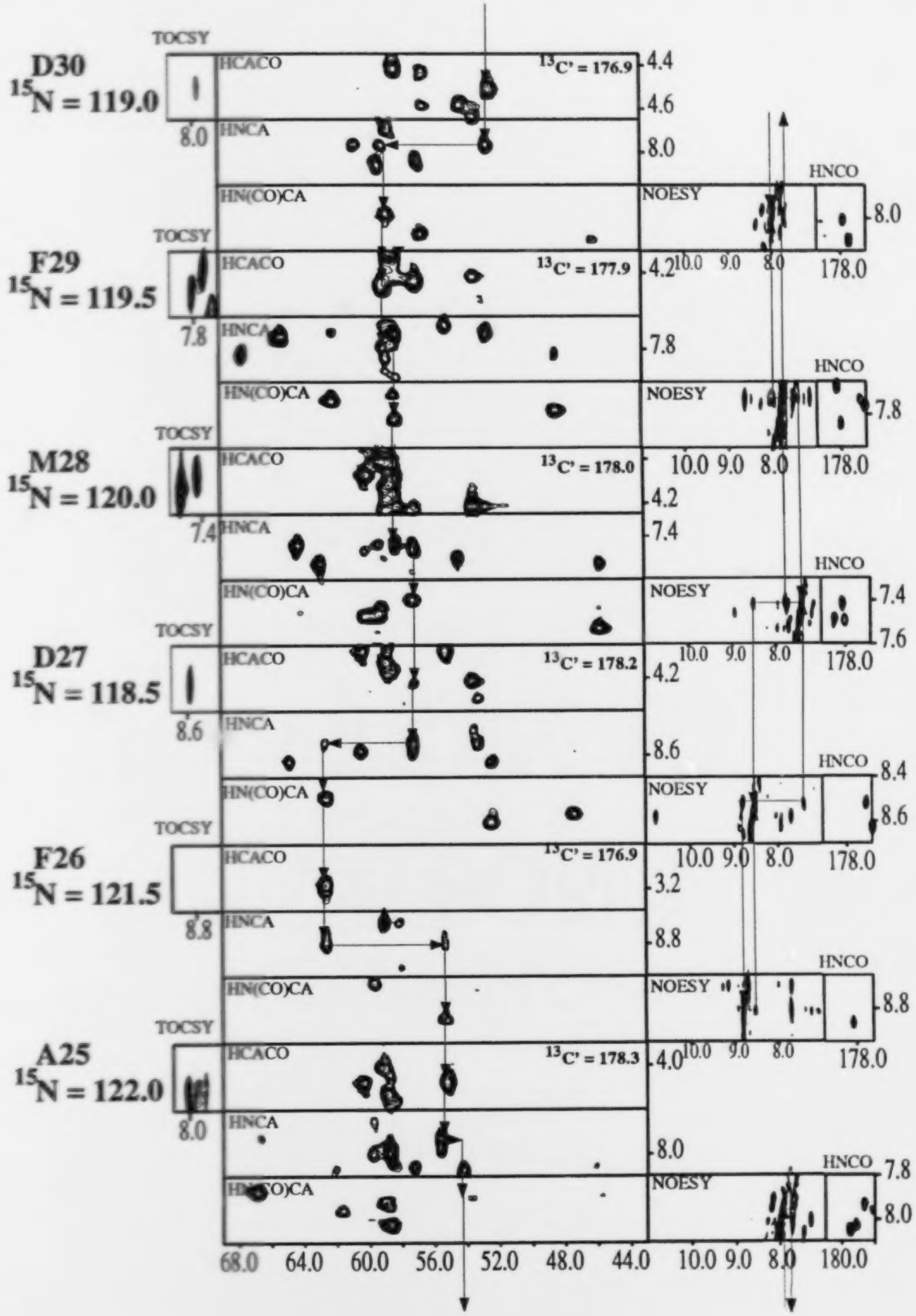

TOCSY
T54
^{15}N = 113.5
8.6
HCACO
^{13}C' = 175.5
4.4
4.6
HNCA
8.6
HN(CO)CA
NOESY
HNCO
8.4
8.6
10.0 9.0 8.0
178.0
P53
^{15}N = ???.?
HCACO
^{13}C' = 177.7
4.8

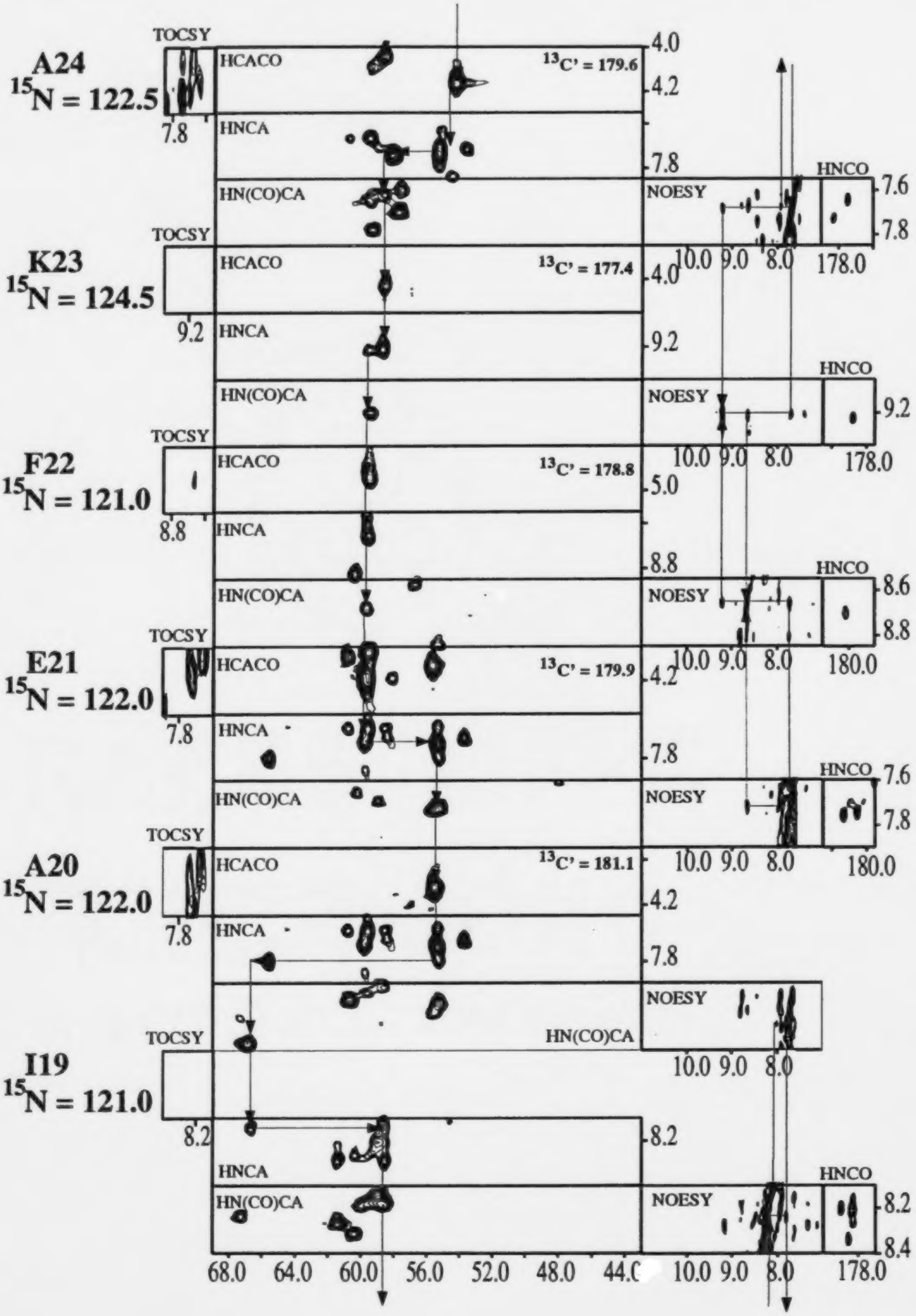

TOCSY
N52
^{15}N = 118.0
8.6
HCACO
^{13}C' = 172.2
5.2
HNCA
8.6
HN(CO)CA
NOESY
HNCO
8.6
10.0 9.0 8.0
174.0
TOCSY
Q51
^{15}N = 120.0
8.2
HCACO
^{13}C' = 174.4
4.6
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.0
8.2
10.0 9.0 8.0
174.0
TOCSY
G50
^{15}N = 108.5
7.8
HCACO
^{13}C' = 174.8
3.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.8
8.0
10.0 9.0 8.0
178.0
TOCSY
L49
^{15}N = 119.0
7.6
HCACO
^{13}C' = 177.7
4.4
HNCA
7.6
HN(CO)CA
NOESY
HNCO
7.6
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
178.0

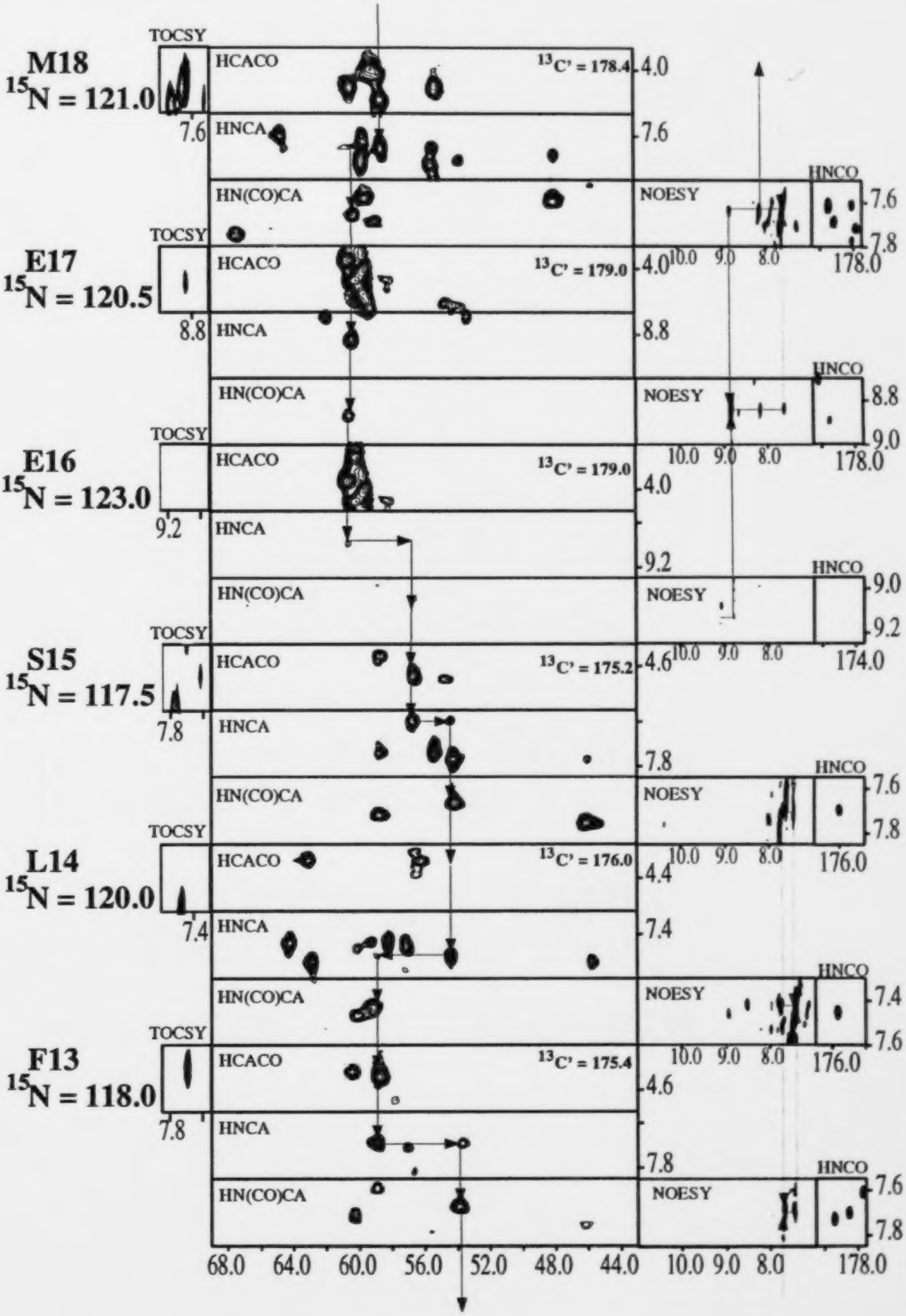

TOCSY
M48
^{15}N = 119.0
HCACO
^{13}C' = 177.7
4.2
7.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.6
7.8
10.0 9.0 8.0
182.0
R47
^{15}N = 120.0
HCACO
^{13}C' = 181.4
4.8
8.0
HNCA
8.0
HN(CO)CA
NOESY
HNCO
8.0
8.2
10.0 9.0 8.0
178.0
M46
^{15}N = 117.5
HCACO
^{13}C' = 179.0
4.0
8.2
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.2
8.4
10.0 9.0 8.0
178.0
V45
^{15}N = 123.0
^{13}C' = 177.8
HCACO
3.6
7.6
HNCA
HN(CO)CA
NOESY
HNCO
7.4
7.6
10.0 9.0 8.0
176.0
T44
^{15}N = 119.5
^{13}C' = 176.3
HCACO
4.0
7.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.8
10.0 9.0 8.0
176.0
G43
^{15}N = 106.5
HCACO
^{13}C' = 175.4
3.8
8.6
HNCA
8.6
HN(CO)CA
NOESY
HNCO
8.6
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
178.0

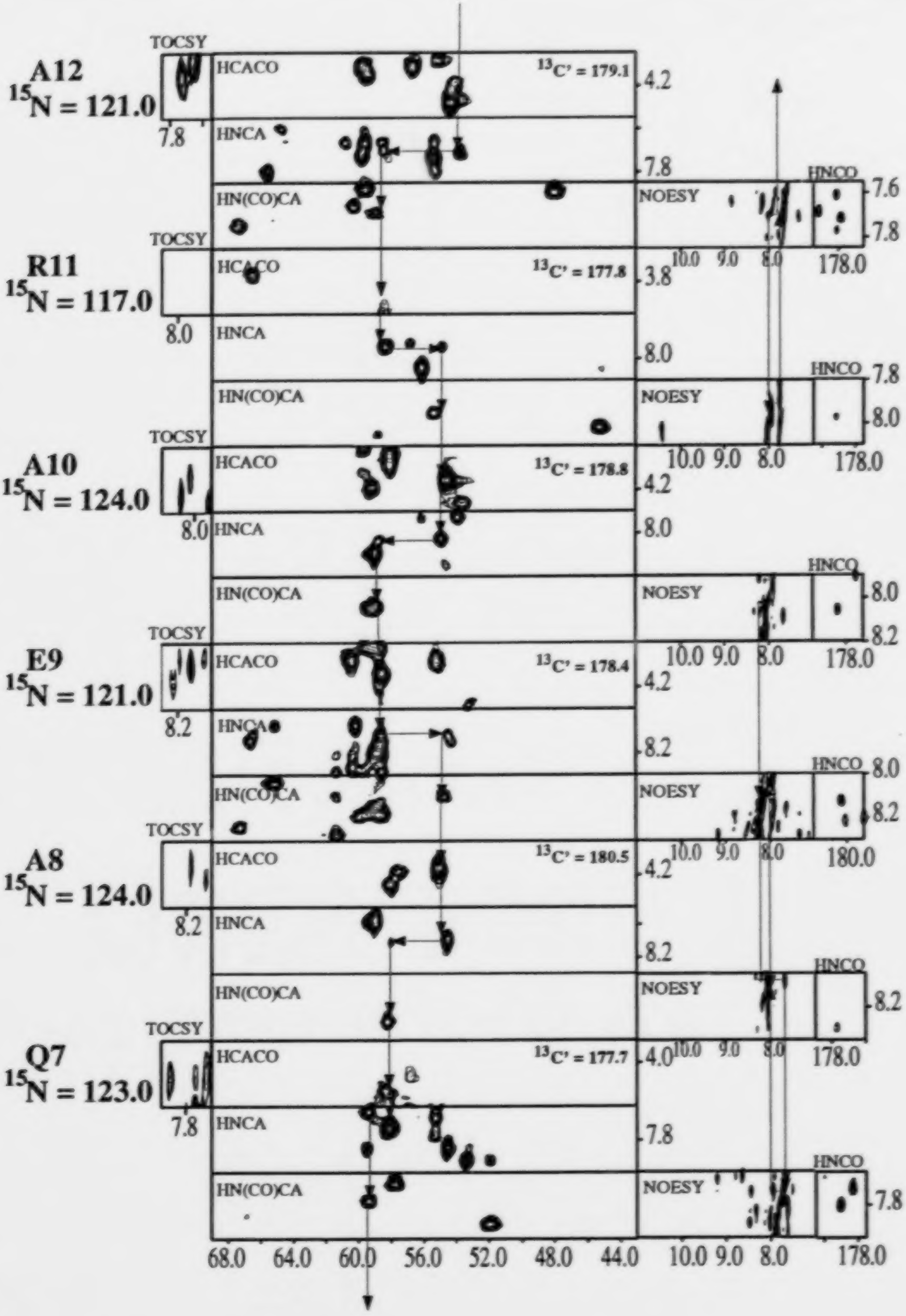

L42
^{15}N = 122.0
TOCSY
HCACO
^{13}C' = 178.8
HNCA
HN(CO)CA
NOESY
HNCO
E41
^{15}N = 122.5
TOCSY
HCACO
^{13}C' = 179.2
HNCA
HN(CO)CA
NOESY
HNCO
K40
^{15}N = 122.5
TOCSY
HCACO
^{13}C' = 179.9
HNCA
HN(CO)CA
NOESY
HNCO
T39
^{15}N = 116.0
TOCSY
HCACO
^{13}C' = 177.7
HNCA
HN(CO)CA
NOESY
HNCO
S38
^{15}N = 124.0
TOCSY
HCACO
^{13}C' = 175.9
HNCA
HN(CO)CA
NOESY
HNCO
I37
^{15}N = 126.5
TOCSY
HCACO
^{13}C' = 176.1
HNCA
HN(CO)CA
NOESY
HNCO
8.6
7.8
8.0
9.2
8.8
9.6
4.0
4.2
8.6
8.8
7.6
7.8
8.0
3.8
9.0
9.2
4.8
8.8
8.6
5.0
9.6
9.8
10.0 9.0 8.0
180.0
178.0
176.0
174.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0

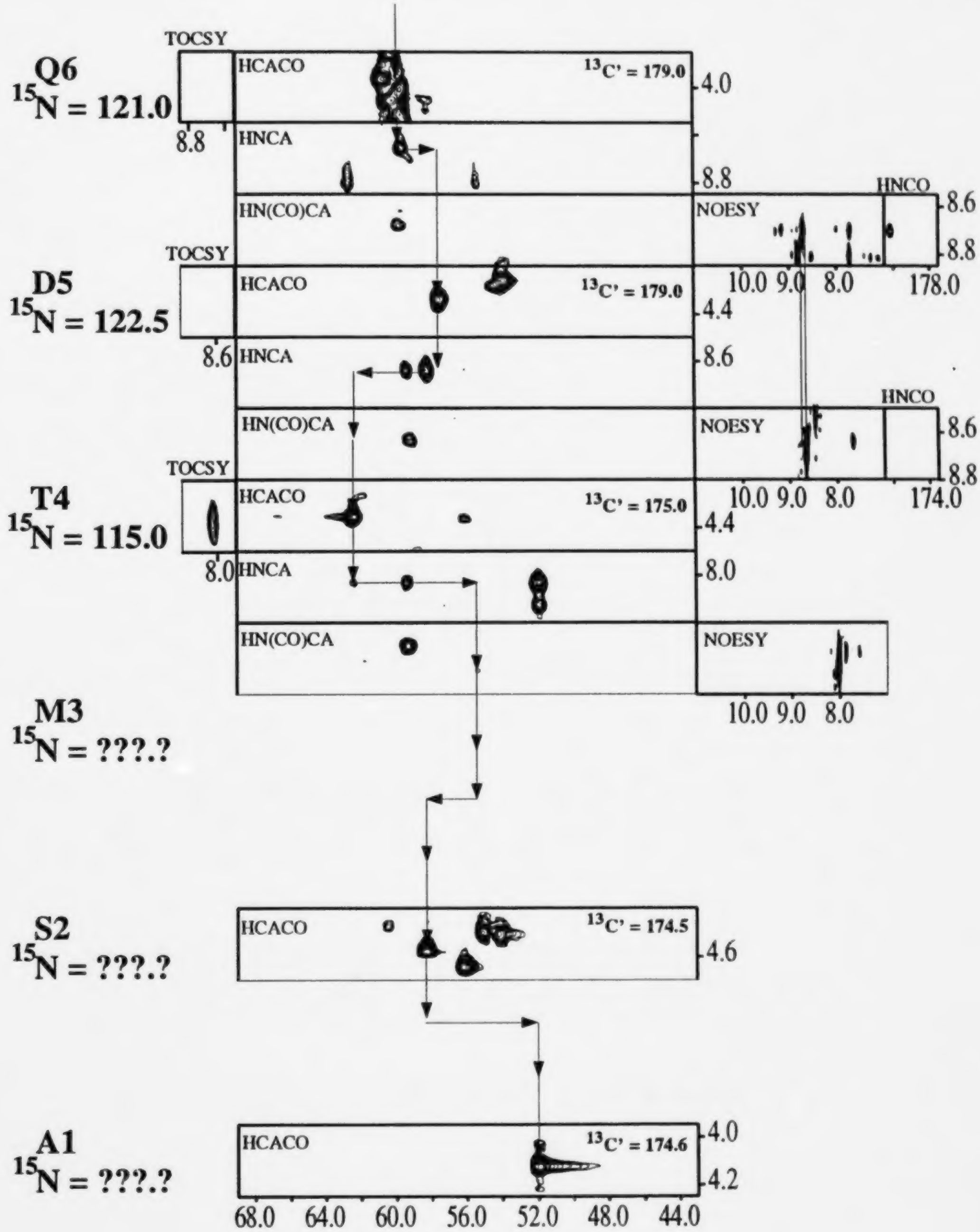

D36
^{15}N = 116.5
TOCSY
HCACO
^{13}C' = 173.4
HNCA
HN(CO)CA
NOESY
HNCO
G35
^{15}N = 114.0
^{13}C' = 173.3
G34
^{15}N = 109.5
^{13}C' = 176.1
G33
^{15}N = 111.5
^{13}C' = 175.9
D32
^{15}N = 114.5
^{13}C' = 177.9
A31
^{15}N = 131.0
^{13}C' = 179.3
5.0
5.2
7.8
4.0
10.6
10.8
8.2
8.0
3.8
4.6
8.4
4.2
7.6
10.0
9.0
174.0
176.0
178.0
68.0
64.0
60.0
56.0
52.0
48.0
44.0


D30
^{15}N = 119.0
TOCSY
HCACO
^{13}C' = 176.9
4.4
4.6
8.0
HNCA
8.0
HN(CO)CA
NOESY
HNCO
8.0
10.0 9.0 8.0
178.0
F29
^{15}N = 119.5
TOCSY
HCACO
^{13}C' = 177.9
4.2
7.8
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.8
10.0 9.0 8.0
178.0
M28
^{15}N = 120.0
TOCSY
HCACO
^{13}C' = 178.0
4.2
7.4
HNCA
7.4
HN(CO)CA
NOESY
HNCO
7.4
7.6
10.0 9.0 8.0
178.0
D27
^{15}N = 118.5
TOCSY
HCACO
^{13}C' = 178.2
4.2
8.6
HNCA
8.6
HN(CO)CA
NOESY
HNCO
8.4
8.6
10.0 9.0 8.0
178.0
F26
^{15}N = 121.5
TOCSY
HCACO
^{13}C' = 176.9
3.2
8.8
HNCA
8.8
HN(CO)CA
NOESY
HNCO
8.8
10.0 9.0 8.0
178.0
A25
^{15}N = 122.0
TOCSY
HCACO
^{13}C' = 178.3
4.0
8.0
HNCA
8.0
HN(CO)CA
NOESY
HNCO
7.8
8.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
180.0


A24
^{15}N = 122.5
K23
^{15}N = 124.5
F22
^{15}N = 121.0
E21
^{15}N = 122.0
A20
^{15}N = 122.0
I19
^{15}N = 121.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 179.6
^{13}C' = 177.4
^{13}C' = 178.8
^{13}C' = 179.9
^{13}C' = 181.1
7.8
9.2
8.8
8.2
4.0
4.2
5.0
7.6
8.6
8.4
10.0 9.0 8.0
178.0
180.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0


M18
^{15}N = 121.0
E17
^{15}N = 120.5
E16
^{15}N = 123.0
S15
^{15}N = 117.5
L14
^{15}N = 120.0
F13
^{15}N = 118.0
TOCSY
HCACO
HNCA
HN(CO)CA
NOESY
HNCO
^{13}C' = 178.4
^{13}C' = 179.0
^{13}C' = 179.0
^{13}C' = 175.2
^{13}C' = 176.0
^{13}C' = 175.4
7.6
8.8
9.2
7.8
7.4
7.8
4.0
7.6
4.0
8.8
4.0
9.2
4.6
7.8
4.4
7.4
4.6
7.8
7.6
7.8
8.8
9.0
9.0
9.2
7.6
7.8
7.4
7.6
7.6
7.8
10.0 9.0 8.0
178.0
178.0
174.0
176.0
176.0
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
178.0


TOCSY
A12
^{15}N = 121.0
7.8
HCACO
^{13}C' = 179.1
4.2
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.6
7.8
10.0 9.0 8.0
178.0
TOCSY
R11
^{15}N = 117.0
8.0
HCACO
^{13}C' = 177.8
3.8
HNCA
8.0
HN(CO)CA
NOESY
HNCO
7.8
8.0
10.0 9.0 8.0
178.0
TOCSY
A10
^{15}N = 124.0
8.0
HCACO
^{13}C' = 178.8
4.2
HNCA
8.0
HN(CO)CA
NOESY
HNCO
8.0
8.2
10.0 9.0 8.0
178.0
TOCSY
E9
^{15}N = 121.0
8.2
HCACO
^{13}C' = 178.4
4.2
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.0
8.2
10.0 9.0 8.0
180.0
TOCSY
A8
^{15}N = 124.0
8.2
HCACO
^{13}C' = 180.5
4.2
HNCA
8.2
HN(CO)CA
NOESY
HNCO
8.2
10.0 9.0 8.0
178.0
TOCSY
Q7
^{15}N = 123.0
7.8
HCACO
^{13}C' = 177.7
4.0
HNCA
7.8
HN(CO)CA
NOESY
HNCO
7.8
68.0 64.0 60.0 56.0 52.0 48.0 44.0
10.0 9.0 8.0
178.0


TOCSY
Q6
15N = 121.0
HCACO
13C' = 179.0
4.0
8.8
HNCA
8.8
HN(CO)CA
NOESY
HNCO
8.6
8.8
10.0 9.0 8.0
178.0
TOCSY
D5
15N = 122.5
HCACO
13C' = 179.0
4.4
8.6
HNCA
8.6
HN(CO)CA
NOESY
HNCO
8.6
8.8
10.0 9.0 8.0
174.0
TOCSY
T4
15N = 115.0
HCACO
13C' = 175.0
4.4
8.0
HNCA
8.0
HN(CO)CA
NOESY
10.0 9.0 8.0
M3
15N = ???.?
S2
15N = ???.?
HCACO
13C' = 174.5
4.6
A1
15N = ???.?
HCACO
13C' = 174.6
4.0
4.2
68.0 64.0 60.0 56.0 52.0 48.0 44.0

Appendix C:

XPLOR Input Files:

1. Simulated Annealing:

```
remarks file  nmr/sa.inp
remarks  Simulated annealing protocol for NMR structure determination.
remarks  The starting structure for this protocol can be any structure with
remarks  a reasonable geometry, such as randomly assigned torsion angles or
remarks  extended strands.  This macro is updated compared to the previous
remarks  macros, a attractive LJ potential has been introduced (by KSK)

{====>}
evaluate ($init_t = 1000 )      {*Initial simulated annealing temperature.*}
{====>}
evaluate ($high_steps= 6000 )       {*Total number of steps at high temp.*}
{====>}
evaluate ($cool_steps = 3000 )     {*Total number of steps during cooling.*}

parameter                          {*Read the parameter file.*}
{====>}
  @../parallhdg.pro
end

{====>}
structure @../tnc_model.psf end            {*Read the structure file.*}

{====>}
coordinates @../tnc_model.pdb              {*Read the coordinates.*}

noe
{====>}
 set echo=off message=off end
  nres=5000          {*Estimate greater than the actual number of NOEs.*}
  class all
{====>}
  @restraints/tnc_rst_7.tbl          {*Read NOE distance ranges.*}
!  @tnc_hbond.tbl                                    {*Read H-bonded distances *}
! set echo=on message=on end
end

{====>}
set message=off echo=off end
restraints dihedral reset
  @phi.tbl
  @psi.tbl
 end
set message=on echo=on end
```

```
{====>}
          {*If protein contains S-S bridges, appropriately modify and *}
          {*then uncomment the following lines. The S-S covalent bonds*}
          {*will be represented as fake NOE distances.               *}
!noe
!  assign (resid  7 and name sg) (resid 34 and name sg)  2.02 0.1 0.1
!  assign (resid  9 and name sg) (resid 50 and name sg)  2.02 0.1 0.1
!end


flags exclude * include bonds angle impr vdw noe cdih end


               {*Friction coefficient for MD heatbath, in 1/ps.   *}
vector do (fbeta=10) (all)
               {*Uniform heavy masses to speed molecular dynamics.*}
vector do (mass=100) (all)

noe                    {*Parameters for NOE effective energy term.*}
 ceiling=1000
 averaging  * cent
 potential  * soft
 scale      * 50.
 sqoffset   * 0.0
 sqconstant * 1.0
 sqexponent * 2
 soexponent * 1
 asymptote  * 0.1                {*Initial value--modified later.*}
 rswitch    * 0.5
end

parameter               {*Parameters for the repulsive energy term.*}
   nbonds
     repel=1.0              {*Initial value for repel--modified later.*}
     rexp=2 irexp=2 rcon=1.
     nbxmod=3
     wmin=0.01
     cutnb=4.5 ctonnb=2.99 ctofnb=3.
     tolerance=0.5
   end
end

restraints dihedral
     scale=5.
end

{====>}
evaluate ($end_count=50)        {*Loop through a family of 30 structures.*}
coor copy end

evaluate ($count = 0)
while ($count < $end_count ) loop main
  evaluate ($count=$count+1)
   coor swap end
   coor copy end

     {* ============================================ Initial minimization.*}
   restraints dihedral   scale=5.   end
   noe asymptote * 0.1  end
   parameter  nbonds repel=1.0   end end
   constraints interaction
```

```
     (all) (all) weights * 1  vdw 0.002 end end
minimize powell nstep=600 drop=10.  nprint=25 end


 {* ======================================== High-temperature dynamics.*}
restraints dihedral   scale=5.   end
constraints interaction
     (all) (all)
      weights * 1  angl 1.0  impr 0.4 bond 1.00 vdw 0.002 end end

evaluate ($nstep1=int($high_steps * 2. / 3. ) )
evaluate ($nstep2=int($high_steps * 1. / 3. ) )

dynamics  verlet
  nstep=$nstep1  timestep=0.005  iasvel=maxwell  firstt=$init_t
  tcoupling=true  tbath=$init_t  nprint=50  iprfrq=0
end


 {* ============== Tilt the asymptote and increase weights on geometry.*}
noe asymptote * 1.0  end

constraints interaction
     (all) (all)
      weights * 1  vdw 0.002  end end
dynamics  verlet
  nstep=$nstep2  timestep=0.005   iasvel=current   tcoupling=true
  tbath=$init_t  nprint=50  iprfrq=0
end

 {* ================================================= Cool the system.*}

restraints dihedral   scale=200.   end

evaluate ($final_t = 100)      { K }
evaluate ($tempstep = 50)      { K }

evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
evaluate ($nstep = int($cool_steps/$ncycle))

evaluate ($ini_rad  = 1.0)       evaluate ($fin_rad  = 0.8)
evaluate ($ini_con=  0.003)       evaluate ($fin_con=  4.0)

evaluate ($bath  = $init_t)
evaluate ($k_vdw = $ini_con)
evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
evaluate ($radius=    $ini_rad)
evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

evaluate ($i_cool = 0)
while ($i_cool < $ncycle) loop cool
  evaluate ($i_cool=$i_cool+1)

  evaluate ($bath  = $bath  - $tempstep)
  evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
  evaluate ($radius=max($fin_rad,$radius*$radfact))

  parameter  nbonds repel=$radius   end end
  constraints interaction
     (all) (all)
      weights * 1. vdw $k_vdw end end
  dynamics  verlet
   nstep=$nstep time=0.005 iasvel=current firstt=$bath
   tcoup=true tbath=$bath nprint=$nstep iprfrq=0
  end


{===>}                                  {*Abort condition.*}
  evaluate ($critical=$temp/$bath)
  if ($critical >  10. ) then
   display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
   stop
  end if

 end loop cool

 {* ======================================= Final minimization.*}

 constraints interaction
    (all) (all)
    weights * 1 vdw 4. end end
 minimize powell nstep=500 drop=10.0 nprint=25 end
parameter  nbonds
      repel=0.0
      VSWI SHIF
      CTOFNB=7.5 CTONNB=6.5 CUTNB=8.5
    end end
 constraints interaction
    (all) (all)
    weights * 1 vdw 1.2 bond 1.0 angl 1.0 end end
 minimize powell nstep=400 drop=10.0 nprint=25 end

 {* ================================= Write out the final structure(s).*}
 print threshold=0.3 noe
 evaluate ($rms_noe=$result)
 evaluate ($violations_noe=$violations)
 print threshold=3. cdih
 evaluate ($rms_cdih=$result)
 evaluate ($violations_cdih=$violations)
 print thres=0.05 bonds
 evaluate ($rms_bonds=$result)
 print thres=3. angles
 evaluate ($rms_angles=$result)
 print thres=5. impropers
 evaluate ($rms_impropers=$result)
 remarks ============================================================
 remarks          overall,bonds,angles,improper,vdw,noe,cdih
 remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
 remarks ============================================================
 remarks          bonds,angles,impropers,noe,cdih
 remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
 remarks ============================================================
 remarks            noe,  cdih
 remarks violations.: $violations_noe, $violations_cdih
 remarks ============================== ============================

{===>}                  {*Name(s) of the family of final structures.*}
 evaluate ($filename="sa_run27_"+encode($count)+".pdb")
 write coordinates output =$filename end

end loop main
stop
```

2. Sub-embedding:

```
remarks file nmr/dg_sub_embed.inp --
remarks        Bound smoothing, (sub)structure embedding,
remarks        and regularization to produce a family of
remarks        DG structures.
remarks
remarks Author: Axel T. Brunger

{===>}
structure @tnc_ca4.psf end                 {*Read structure file.*}


parameter
{===>}
  @parallhdg.pro                      {*Read parameters.*}
  nbonds
    repel = 0.75             {*This scales the van der Waals radii.*}
  end
end

noe
{===>}
 set echo-off message=off end
  nres=5000                  {*Approximate number greater than the*}
                         {*actual number of NOEs.         *}
  class = all

{===>}
  @tnc_rst_new23.tbl                {*Read NOE distance ranges.  *}
  @calcium.tbl                 {*Note that no other settings *}
                           {*are important for embedding.*}
end


{===>}
set message=off echo=off end
restraints dihedral reset
 @phi.tbl                    {*Read dihedral angle restraints.*}
 @psi.tbl                    {*Read dihedral angle restraints.*}
 @chi.tbl                    {*Read dihedral angle restraints.*}
 end
set message=on echo=on end


       {*Read template for pseudoatom correction and for target values*}
       {*for conformational constraints (bonds, angles, etc.).      *}
{===>} coor disp=refe @tnc_ca4_rnd.pdb


{===>}
                   {*Store (sub)structure selection in store1.*}
     {*The following substructure selection is typical for a protein;  *}
     {*for nucleic acids try name p or name c3' or name c5' or name c1'*}
     {*or name n9 or name n1 or name c2 or name c4 or name n3.       *}

vector ident ( store1 ) (name ca or name ha or name n or name hn
                     or name c or name cb* or name cg*)

                    {*Energy flags: both NOEs and dihedral*}
                    {*angle restraints are included.    *}
```

```
flags exclude * include bond angle dihedral improper vdw noe cdih end

mmdg                              {*Create bounds matrix.*}
  reference=coordinates
  storebounds                       {*Store bounds matrix.*}
end

                         {*Include DG term for regularization.*}
flags exclude * include dg end
constraints interaction=( recall1 ) ( recall1 ) end

evaluate ($count = 0)

          {*The following loop produces a family of 100 substructures.*}
{===>}
while ($count < 100 ) loop main

  evaluate ($count=$count+1)
  evaluate ($embedded = false)

                         {*Loop until embedding is successful;*}
                         {*normally the success rate is high, *}
                         {*and it will work during the first  *}
                         {*pass.                              *}
  while ($embedded = false) loop embed
    mmdg
      recallbounds                      {*Get bounds matrix.*}

      substructure=( recall1 )

      selection=( recall1 )            {*Specify parameters    *}
      scale=100. exponent=2               {*for DG-regularization.*}
    end
  end loop embed

  vector do (x = x * $dgscale) (known)          {*Scale the structure;  *}
  vector do (y = y * $dgscale) (known)          {*the symbol $DGSCALE is*}
  vector do (z = z * $dgscale) (known)          {*defined by MMDG.      *}

  minimize powell                            {*Regularization.*}
    nstep=100 drop=10. nprint=25
  end

{===>}       {*Uncomment the following lines if a test for the correct *}
          {*enantiomer is desired based on improper energy.          *}
          {*For this test to be successful, the substructures must  *}
          {*contain chiral centers (e.g., CA) and all their ligands.*}
!flags exclude dg include impr end
!energy end
!evaluate ($old_e=$impr)
!vector do (x=-x) ( known )
!energy end
!if ($impr > $old_e) then
!  vector do (x=-x) ( known )
!end if
!flags exclude impr include dg end

{===>}       {* Uncomment the following lines if a test for the correct*}
          {* enantiomer is desired based on an rms difference from a*}
          {* reference structure.                                   *}
!coor disp=comp @reference.pdb              {*Read reference structure.*}
```

```
!coor fit sele=( known ) end
!coor rms sele=( known ) end
!evaluate ($old_rms=$result)
!vector do (x=-x) ( known )
!coor fit sele=( known ) end
!coor rms sele=( known ) end
!if ($result > $old_rms) then
!  vector do (x=-x) ( known )
!end if

  remarks  produced by nmr/dg_sub_embed.inp

{====>}                {*Name(s) of the family of embedded substructures.*}
  evaluate ($filename="dg_sub_embed_"+encode($count)+".pdb")

  write coordinates output =$filename end

end loop main

stop
```

3. Distance geometry / simulated annealing:

```
remarks file nmr/dgsa.inp  -- Simulated annealing regularization
remarks                  and refinement for embedded distance
remarks                  geometry structures or substructures.
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger


{====>}
evaluate ($init_t = 2000)          {*Initial annealing temperature, in K.*}
{====>}
evaluate ($high_steps = 2000 )       {*Total number of steps at high temp.*}
{====>}
evaluate ($cool_steps = 2000 )       {*Total number of steps for cooling.*}

parameter                           {*Read the parameter file.*}
{====>}
  @parallhdg.pro
end

{====>}
structure @tnc_ca4.psf end                {*Read the structure file.*}

noe
{====>}
set echo=off message=off end
  nres=5000          {*Estimate greater than the actual number of NOEs.*}
  class all
{====>}
  @tnc_rst_new23.tbl                   {*Read NOE distance ranges.*}
  @calcium.tbl                       {*Read NOE distance ranges.*}
end


restraints dihedral reset
  @phi.tbl
  @psi.tbl
  @chi.tbl
end
```

```
set message=on echo=on end


vector do (fbeta=10) (all) {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (mass=100) (all)           {*Uniform heavy masses to speed MD.*}

noe                        {*Parameters for NOE effective energy term.*}
  ceiling=1000
  averaging  * cent
  potential  * square
  sqconstant * 1.
  sqexponent * 2
  scale      * 50.          {*Constant NOE scale throughout the protocol.*}
end

parameter                  {*Parameters for the repulsive energy term.*}
  nbonds
    repel=0.5              {*Initial value for repel--modified later.*}
    rexp=2 irexp=2 rcon=1.
    nbxmod=-2              {*Initial value for nbxmod--modified later.*}
    wmin=0.01
    cutnb=4.5 ctonnb=2.99 ctofnb=3.
    tolerance=0.5
  end
end

restraints dihedral
    scale=5.                        {*Initial weight--modified later.*}
end

{====>}
evaluate ($end_count=100)        {*Loop through a family of 100 structures.*}

evaluate ($count = 57)
while ($count < $end_count ) loop main

  evaluate ($count=$count+1)

{====>}                         {*Filename(s) for embedded coordinates.*}
  evaluate ($filename="sub_embedding/dg_sub_embed_"+encode($count)+".pdb")

                      {*Test for the correct enantiomer;          *}
{====>}                      {*if you want to bypass this test because the *}
                      {*substructures were tested previously, simply*}
                      {*remove the -1 from the next statement.      *}
for $image in ( 1 -1 ) loop imag
    coor initialize end
    coor @@$filename
    vector do (x=x * $image) ( known )
    vector identity (store1) (not known)       {*Set store1 to unknowns.*}

    {* ================== Create local ideal geometry by template fitting;*}
    {*                  this also takes care of unknown atoms.          *}

    set message=off echo=off end
    coor copy end         {*Store current coordinates in comparison set.*}

{====>}                  {*The user has to supply a template coordinate set.*}
    coor @@tnc_ca4_rnd.pdb

    for $id in id ( tag ) loop fit            {*Loop over residue tags.*}
    coordinates              {*LSQ fitting using known coordinates.*}
      fit select = ( byresidue (id $id) and not store1 )
    end
                           {*Store fitted template coordinates for this residue.*}
   coor copy selection=( byresidue (id $id) ) end

 end loop fit

 coor swap end
 set message=on echo=on end

{* ============================== Minimization of bonds, VDWs, and NOEs.*}
 restraints dihedral   scale=5.   end
 parameter  nbonds   nbxmod=-2 repel=0.5   end  end
 flags exclude * include bond vdw noe cdih ncs end
 constraints interaction (all) (all) weights * 1.  vdw 20. end end

 minimize powell nstep=100 nprint=10 end

{* ================================================== Include angles. *}

 flags include angl end

 minimize powell nstep=100 nprint=10 end

{* ============ Dynamics, slowly introducing chirality and planarity. *}
 flags include impr end

 evaluate ($nstep1 = int($high_steps/8))
 evaluate ($nstep2 = int($high_steps/2))

 constraints inter (all) (all) weights * 0.1 impr 0.05 vdw 20. end end
 dynamics  verlet
   nstep=$nstep1 time=0.001 iasvel=maxwell firstt=$init_t
   tcoup=true tbath=$init_t nprint=100 iprfrq=0
 end
 constraints inter (all) (all) weights * 0.2 impr 0.1  vdw 20. end end
 dynamics  verlet
   nstep=$nstep1 time=0.001 iasvel=current firstt=$init_t
   tcoup=true tbath=$init_t nprint=100 iprfrq=0
 end

 parameter  nbonds repel=0.9   end  end
 constraints inter (all) (all) weights * 0.2 impr 0.2 vdw 0.01 end end
 dynamics  verlet
   nstep=$nstep1 time=0.001 iasvel=current firs tt=$init_t
   tcoup=true tbath=$init_t nprint=100 iprfrq=0
 end

 parameter nbonds nbxmod=-3  end  end
 constraints inter (all) (all) weights * 0.4 impr 0.4 vdw 0.003 end end
 dynamics  verlet
   nstep=$nstep2 time=0.001 iasvel=current firstt=$init_t
   tcoup=true tbath=$init_t nprint=100 iprfrq=0
 end

 constraints inter (all) (all) weights * 1.0 impr 1.0 vdw 0.003 end end
 dynamics  verlet
   nstep=$nstep1 time=0.001 iasvel=current  firstt=$init_t
   tcoup=true tbath=$init_t nprint=100 iprfrq=0
 end
```

```
  if ($image = 1) then
    vector do (store7=x) ( all )        {*Store first image in stores.*}
    vector do (store8=y) ( all )
    vector do (store9=z) ( all )
    vector do (store4=vx) ( all )
    vector do (store5=vy) ( all )
    vector do (store6=vz) ( all )
  end if

end loop imag

{* ==================== Establish the correct handedness of the structure.*}

energy end
evaluate ($e_minus=$ener)
coor copy end
vector do (x=store7) ( all )
vector do (y=store8) ( all )
vector do (z=store9) ( all )
energy end
evaluate ($e_plus=$ener)
if ( $e_plus > $e_minus ) then
  evaluate ($hand=-1 )
  coor swap end
else
  evaluate ($hand= 1 )
  vector do (vx=store4) ( all )
  vector do (vy=store5) ( all )
  vector do (vz=store6) ( all )
end if


  {* ==================================== Increase VDW interaction and cool.*}

restraints dihedral   scale=200.   end

evaluate ($final_t = 100)    { K }
evaluate ($tempstep = 50)    { K }

evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
evaluate ($nstep = int($cool_steps/$ncycle))

evaluate ($ini_rad  = 0.9)       evaluate ($fin_rad  = 0.75)
evaluate ($ini_con=  0.003)       evaluate ($fin_con=  4.0)
evaluate ($ini_ncs=  0.01)       evaluate ($fin_ncs=  10.0)

evaluate ($bath  = $init_t)
evaluate ($k_vdw = $ini_con)
evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
evaluate ($radius=    $ini_rad)
evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))
evaluate ($wncs =  $ini_ncs)
evaluate ($ncsfact = ($fin_ncs/$ini_ncs)^(1/$ncycle))


evaluate ($i_cool = 0)
while ($i_cool < $ncycle) loop cool
  evaluate ($i_cool=$i_cool+1)

  evaluate ($bath  = $bath  - $tempstep)
  evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
  evaluate ($radius=max($fin_rad,$radius*$radfact))
```

```
      evaluate ($wncs=min($fin_ncs,$wncs*$ncsfact))

      ncs restraints
      group
      weight-ncs=$wncs
      end end


      parameter  nbonds repel=$radius   end end
      constraints interaction (not name SG) (all) weights * 1. vdw $k_vdw end end

      dynamics  verlet
        nstep=$nstep time=0.001 iasvel=current firstt=$bath
        tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


  {===>}                                  {*Abort condition.*}
     evaluate ($critical=$temp/$bath)
     if ($critical >  10. ) then
       display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
       stop
     end if

   end loop cool

   {* ================================================ Final minimization.*}
    constraints interaction
         (all) (all)
         weights * 1. vdw 4. end end
   minimize powell nstep= 200 nprint=25 end

    {* ====================== Analyze and write out the final structure(s).*}
   print threshold=0.5 noe
   evaluate ($rms_noe=$result)
   evaluate ($violations_noe=$violations)
   print threshold=5. cdih
   evaluate ($rms_cdih=$result)
   evaluate ($violations_cdih=$violations)
   print thres=0.05 bonds
   evaluate ($rms_bonds=$result)
   print thres=5. angles
   evaluate ($rms_angles=$result)
   print thres=5. impropers
   evaluate ($rms_impropers=$result)
   remarks ============================================================
   remarks           overall,bonds,angles,improper,vdw,noe,cdih
   remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
   remarks ============================================================
   remarks           bonds,angles,impropers,noe,cdih
   remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
   remarks ============================================================
   remarks             noe,  cdih
   remarks violations.: $violations_noe, $violations_cdih
   remarks ============================= ==============================
   remarks handedness: $hand, enantiomer discrimination ( $e_plus : $e_minus )
 {===>}                   {*Name(s) of the family of final structures.*}
   evaluate ($filename="dgsa_"+encode($count)+".pdb")
   write coordinates output =$filename end
 end loop main
 stop
```

4. Simulated annealing (refinement):

```
remarks file nmr/refine.inp  -- Simulated annealing refinement
remarks                   for NMR structure determination
remarks  Authors: Michael Nilges, John Kuszewski, and Axel T. Brunger

{===>}
evaluate ($init_t = 1000)          {*Initial annealing temperature, in K.*}
{===>}
evaluate ($cool_steps = 8000 )     {*Total number of steps during cooling.*}

parameter                          {*Read the parameter file.*}
{===>}
  @parallhdg.pro
end

{===>} structure @tnc_ca4.psf end            {*The structure file.*}

noe
{===>}
set message=off echo=off end
  nres=5000            {*Estimate greater than the actual number of NOEs.*}
  class all
{===>}
  @tnc_rst_new23.tbl                   {*Read NOE distance ranges.*}
  @calcium.tbl                       {*Read NOE distance ranges.*}
end

{===>}
restraints dihedral reset
    @phi.tbl                   {*Read dihedral angle restraints.*}
    @psi.tbl                   {*Read dihedral angle restraints.*}
    @chi.tbl                   {*Read dihedral angle restraints.*}
end
set message=on echo=on end

                  {*Friction coefficient for MD heatbath, in 1/ps.*}
vector do (fbeta=10) (all)
vector do (mass=100) (all)     {*Heavy masses to speed molecular dynamics.*}

noe                     {*Parameters for NOE effective energy term.*}
  ceiling=1000
  averaging  * cent
  potential  * square
  sqconstant * 1.
  sqexponent * 2
  scale     * 50.       {*Constant NOE scale throughout the protocol.*}
end

parameter                 {*Parameters for the repulsive energy term.*}
  nbonds
    repel=1.00              {*Initial value for repel--modified later.*}
    rexp=2 irexp=2 rcon=1.
    nbxmod=3
    wmin=0.01
    cutnb=4.5 ctonnb=2.99 ctofnb=3.
    tolerance=0.5
  end
end

restraints dihedral
```

```
      scale=200.
  end

  {===>}
  evaluate ($end_count=100)         {*Loop through a family of 10 structures.*}

  evaluate ($count = 0)
  while ($count < $end_count ) loop main

    evaluate ($count=$count+1)

  {===>}                            {*Filename(s) for embedded coordinates.*}
    evaluate ($filename="dis_geo/dgsa_"+encode($count)+".pdb")

    coor @@$filename

    flags exclude * include bond angl impr vdw noe cdih end

    vector do (vx=maxwell($init_t)) ( all )
    vector do (vy=maxwell($init_t)) ( all )
    vector do (vz=maxwell($init_t)) ( all )

    evaluate ($final_t = 100)    { K }
    evaluate ($tempstep = 50)    { K }

    evaluate ($ncycle = ($init_t-$final_t)/$tempstep)
    evaluate ($nstep = int($cool_steps/$ncycle))

    evaluate ($ini_rad  = 0.9)       evaluate ($fin_rad  = 0.75)
    evaluate ($ini_con=  0.003)       evaluate ($fin_con=  4.0)

    evaluate ($bath  = $init_t)
    evaluate ($k_vdw = $ini_con)
    evaluate ($k_vdwfact = ($fin_con/$ini_con)^(1/$ncycle))
    evaluate ($radius=    $ini_rad)
    evaluate ($radfact = ($fin_rad/$ini_rad)^(1/$ncycle))

    evaluate ($i_cool = 0)
    while ($i_cool < $ncycle) loop cool
      evaluate ($i_cool=$i_cool+1)

      evaluate ($bath  = $bath  - $tempstep)
      evaluate ($k_vdw=min($fin_con,$k_vdw*$k_vdwfact))
      evaluate ($radius=max($fin_rad,$radius*$radfact))

      parameter  nbonds repel=$radius   end end
      constraints interaction (all) (all) weights * 1. vdw $k_vdw end end

      dynamics  verlet
        nstep=$nstep time=0.003 iasvel=current firstt=$bath
        tcoup=true tbath=$bath nprint=$nstep iprfrq=0
      end


  {===>}                                     {*Abort condition.*}
      evaluate ($critical=$temp/$bath)
      if ($critical >  10. ) then
        display  ****&&&& rerun job with smaller timestep (i.e., 0.003)
        stop
      end if
    end loop cool
```

```
{* ================================================== Final minimization.*}
minimize powell nstep= 200 nprint=25 end

{* ================================== Write out the final structure(s).*}
print threshold=0.5 noe
evaluate ($rms_noe=$result)
evaluate ($violations_noe=$v iolations)
print threshold=5. cdih
evaluate ($rms_cdih=$result)
evaluate ($violations_cdih=$violations)
print thres=0.05 bonds
evaluate ($rms_bonds=$result)
print thres=5. angles
evaluate ($rms_angles=$result)
print thres=5. impropers
evaluate ($rms_impropers=$result)
remarks ===============================================================
remarks          overall,bonds,angles,improper,vdw,noe,cdih
remarks energies: $ener, $bond, $angl, $impr, $vdw, $noe, $cdih
remarks ===============================================================
remarks          bonds,angles,impropers,noe,cdih
remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
remarks ===============================================================
remarks            noe,  cdih
remarks violations.: $violations_noe, $violations_cdih
remarks ===============================================================

{===>}                    {*Name(s) of the family of final structures.*}
 evaluate ($filename="refine_"+encode($count)+".pdb")

 write coordinates output =$filename end

end loop main

stop
```

5. Structure Analysis:

```
remarks file  nmr/accept.inp
remarks  Analysis of a family of NMR structures--
remarks  generation of a subfamily of "acceptable" structures

parameter                          {*Read the parameter file.*}
{===>}
  @../parallhdg.pro
end

{===>}
  structure @../tnc_model.psf end              {*Read the structure file.*}

set message=off echo=off end
noe
{===>}
  nres=5000            {*Estimate greater than the actual number of NOEs.*}
  class all
{===>}
  @restraints/tnc_rst_7.tbl                  {*Read NOE distance ranges.*}
end

{===>}
```

```
restraints dihedral reset
  @phi.tbl                {*Read dihedral angle restraints.*}
  @psi.tbl                {*Read dihedral angle restraints.*}
end
set message=on echo=on end

noe                     {*Parameters for NOE effective energy term.*}
  ceiling=1000
  averaging  * cent
  potential  * square
  sqconstant * 1.
  sqexponent * 2
  scale      * 50.
end

parameter                  {*Parameters for the repulsive energy term.*}
  nbonds
    repel=0.75
    rexp=2 irexp=2 rcon=4.
    nbxmod=3
    wmin=0.01
    cutnb=4.5 ctonnb=2.99 ctofnb=3.
    tolerance=0.5
  end
end

restraints dihedral
    scale=200.
end

flags exclude * include bonds angle impr vdw noe cdih end

set precision=4 end

{===>}
evaluate ($end_count=50)         {*Loop through a family of 10 structures.*}

evaluate ($accept_count = 0)
evaluate ($count = 0)
while ($count < $end_count ) loop main
  evaluate ($count=$count+1)
{===>}                         {*Filename(s) for embedded coordinates.*}
  evaluate ($filename="run27/sa_run27_"+encode($count)+".pdb")

  coor @@$filename

  evaluate ($accept=0)
                        {*Print all NOE violations larger than 0.3 A *}
                        {*and compute RMS difference between observed*}
                        {*and model distances.                       *}
  print threshold=0.5 noe
  evaluate ($rms_noe=$result)
  evaluate ($violations_noe=$violations)
  if ($violations_noe > 0) then  evaluate ( $accept=$accept + 1)   end if

                             {*Print all dihedral angle restraint*}
                             {*violations.                       *}
  print threshold=5. cdih
  evaluate ($rms_cdih=$result)
  evaluate ($violations_cdih=$violations)
  if ($violations_cdih > 0) then  evaluate ( $accept=$accept + 1)   end if
  print thres=0.05 bonds          {*Print deviations from ideal geometry.*}
  evaluate ($rms_bonds=$result)
  if ($result > 0.01) then  evaluate ( $accept=$accept + 1)   end if

  print thres=5. angles
  evaluate ($rms_angles=$result)
  if ($result > 1) then  evaluate ( $accept=$accept + 1)   end if

  print thres=5. impropers
  evaluate ($rms_impropers=$result)

  distance from=( not hydrogen ) to=( not hydrogen ) cutoff=1.5 end

          {*Acceptance criteria: no NOE violations greater than 0.5 A,*}
          {*no dihedral angle restraint violations > 5 deg,           *}
          {*rms difference for bond deviations from ideality < 0.01 A,*}
          {*rms difference for angle deviations from ideality < 2 deg.*}
  energy end

  if ($accept = 0 ) then
    evaluate ($accept_count=$accept_count+1)

{===>}
    evaluate ($filename="run27_accept/accept_run27_"+encode($accept_count)+".pdb")
    remarks ============================================================
    remarks           overall,bonds,angles,vdw,noe,cdih
    remarks energies: $ener, $bond, $angl, $vdw, $noe, $cdih, $impr
    remarks ============================================================
    remarks           bonds, angles, impropers, noe, cdih
    remarks rms-d: $rms_bonds,$rms_angles,$rms_impropers,$rms_noe,$rms_cdih
    remarks ============================================================
    remarks             noe,  cdih
    remarks violations.: $violations_noe, $violations_cdih

    write coordinates output=$filename end
  end if

end loop main

stop
```

Appendix D:

Restraint Files:

1. Distance Restraints

```
assign (resid  1 and name   HA) (resid  1 and name  HB*) 3.81 2.12 2.11
assign (resid  1 and name  HB*) (resid  2 and name   HA) 4.96 2.90 2.91
assign (resid  2 and name  HB*) (resid  2 and name   HA) 4.78 2.81 2.80
assign (resid  4 and name   HA) (resid  4 and name HG2*) 4.12 2.42 2.42
assign (resid  4 and name   HB) (resid  4 and name   HA) 4.32 2.33 2.33
assign (resid  4 and name   HB) (resid  4 and name HG2*) 4.96 2.91 2.91
assign (resid  4 and name HG2*) (resid 68 and name  HB1) 5.12 3.00 2.99
assign (resid  5 and name  HB2) (resid  5 and name  HB1) 3.83 2.13 2.13
assign (resid  6 and name   HA) (resid 80 and name HG2*) 4.62 2.72 2.72
assign (resid  6 and name   HA) (resid  9 and name  HG2) 3.87 2.09 2.09
assign (resid  6 and name   HA) (resid  6 and name  HB1) 4.19 2.26 2.26
assign (resid  6 and name   HA) (resid  6 and name  HG2) 3.81 2.05 2.06
assign (resid  6 and name  HB1) (resid  4 and name   HA) 4.42 2.39 2.38
assign (resid  6 and name  HB2) (resid  4 and name   HA) 4.41 2.38 2.38
assign (resid  6 and name  HB2) (resid  4 and name   HB) 4.54 2.46 2.46
assign (resid  6 and name  HB2) (resid  6 and name  HG1) 4.43 2.39 2.39
assign (resid  6 and name  HB2) (resid  6 and name  HB1) 3.58 1.88 1.88
assign (resid  6 and name  HB1) (resid 80 and name HG2*) 4.88 2.86 2.86
assign (resid  7 and name   HA) (resid 80 and name HG2*) 4.10 2.40 2.40
assign (resid  8 and name   HA) (resid  8 and name  HB*) 3.76 2.06 2.06
assign (resid  8 and name  HB*) (resid  5 and name   HA) 3.72 2.02 2.02
assign (resid  9 and name  HG1) (resid  9 and name  HG2) 2.90 1.20 1.19
assign (resid  9 and name  HG1) (resid  9 and name   HA) 4.12 2.22 2.21
assign (resid  9 and name  HG2) (resid  9 and name   HA) 4.43 2.39 2.39
assign (resid  9 and name  HG1) (resid  5 and name   HA) 4.43 2.39 2.39
assign (resid  9 and name   HA) (resid 12 and name  HB*) 4.99 2.92 2.92
assign (resid 10 and name   HA) (resid 10 and name  HB*) 3.96 2.26 2.26
assign (resid 10 and name   HA) (resid 83 and name HG2*) 5.19 3.03 3.03
assign (resid 10 and name   HA) (resid 14 and name   HG) 4.62 2.49 2.48
assign (resid 10 and name  HB*) (resid 80 and name   HA) 4.79 2.81 2.82
assign (resid 10 and name  HB*) (resid 83 and name HG2*) 5.21 3.27 3.27
assign (resid 11 and name  HG2) (resid 19 and name HD1*) 4.72 2.77 2.78
assign (resid 12 and name   HA) (resid 12 and name  HB*) 4.02 2.32 2.33
assign (resid 13 and name  HB1) (resid 13 and name   HA) 4.25 2.29 2.29
assign (resid 13 and name  HB2) (resid 13 and name   HA) 4.20 2.27 2.26
assign (resid 14 and name   HG) (resid 13 and name  HB2) 4.47 2.41 2.41
assign (resid 14 and name   HG) (resid 14 and name  HB1) 3.88 2.09 2.09
assign (resid 14 and name   HG) (resid 14 and name  HB2) 3.60 1.91 1.90
assign (resid 14 and name   HG) (resid 14 and name HD1*) 4.56 2.69 2.70
assign (resid 14 and name   HG) (resid 14 and name HD2*) 4.47 2.64 2.65
assign (resid 14 and name   HA) (resid 18 and name  HB1) 4.17 2.25 2.25
assign (resid 14 and name HD1*) (resid 14 and name  HB1) 4.06 2.36 2.36
assign (resid 14 and name HD1*) (resid 11 and name   HA) 4.39 2.60 2.60
assign (resid 14 and name HD1*) (resid 14 and name  HB2) 4.17 2.46 2.46
assign (resid 14 and name HD2*) (resid 14 and name  HB2) 4.73 2.78 2.79
assign (resid 14 and name HD2*) (resid 82 and name  HB1) 4.97 2.91 2.92
assign (resid 14 and name HD2*) (resid 14 and name   HA) 4.05 2.35 2.35
assign (resid 14 and name HD2*) (resid 86 and name  HE*) 5.32 3.33 3.33
assign (resid 15 and name   HA) (resid 15 and name  HB1) 3.60 1.91 1.90
assign (resid 15 and name   HA) (resid 15 and name  HB2) 3.75 2.02 2.02
assign (resid 15 and name   HA) (resid 18 and name  HB1) 4.23 2.28 2.29
assign (resid 15 and name  HB1) (resid 15 and name  HB2) 3.38 1.68 1.68
assign (resid 16 and name   HA) (resid 19 and name HD1*) 4.63 2.72 2.73
assign (resid 16 and name   HA) (resid 19 and name   HB) 3.87 2.09 2.09
assign (resid 17 and name  HB2) (resid 17 and name   HA) 4.61 2.49 2.49
assign (resid 17 and name  HB1) (resid 17 and name  HG1) 4.25 2.29 2.29
assign (resid 17 and name  HB2) (resid 17 and name  HG1) 4.25 2.29 2.29
assign (resid 17 and name  HB2) (resid 17 and name  HG2) 3.54 1.84 1.84
assign (resid 17 and name  HB1) (resid 17 and name   HA) 4.00 2.16 2.16
assign (resid 17 and name  HB1) (resid 17 and name  HG2) 3.55 1.85 1.85
assign (resid 17 and name  HG1) (resid 17 and name   HA) 4.33 2.33 2.33
assign (resid 17 and name  HG2) (resid 17 and name   HA) 4.19 2.26 2.25
assign (resid 17 and name  HG2) (resid 17 and name  HG1) 3.55 1.85 1.85
assign (resid 17 and name   HA) (resid 20 and name  HB*) 4.35 2.58 2.58
assign (resid 17 and name  HB2) (resid 18 and name   HA) 3.64 1.94 1.94
assign (resid 18 and name  HB2) (resid 18 and name   HA) 4.12 2.22 2.22
assign (resid 18 and name  HG1) (resid 18 and name   HA) 4.69 2.53 2.52
assign (resid 18 and name  HB2) (resid 14 and name  HB2) 3.81 2.05 2.05
assign (resid 18 and name  HG1) (resid 18 and name  HB2) 4.34 2.34 2.34
assign (resid 18 and name  HG1) (resid 18 and name  HE*) 4.67 2.75 2.75
assign (resid 18 and name  HG1) (resid 18 and name  HB1) 4.19 2.26 2.26
assign (resid 18 and name  HG2) (resid 18 and name  HG1) 3.36 1.66 1.66
assign (resid 18 and name  HB2) (resid 14 and name   HA) 4.40 2.37 2.37
assign (resid 18 and name  HB1) (resid 18 and name   HA) 4.22 2.27 2.28
assign (resid 19 and name HG12) (resid 19 and name   HA) 4.60 2.48 2.47
assign (resid 19 and name HG11) (resid 19 and name   HB) 4.01 2.16 2.16
assign (resid 19 and name HG11) (resid 14 and name  HB1) 4.41 2.38 2.38
assign (resid 19 and name HG12) (resid 14 and name  HB1) 3.88 2.09 2.09
assign (resid 19 and name HG12) (resid 19 and name HG11) 3.68 1.98 1.98
assign (resid 19 and name HG11) (resid 19 and name HG2*) 4.21 2.50 2.50
assign (resid 19 and name HG11) (resid 19 and name HD1*) 3.98 2.28 2.28
assign (resid 19 and name HG11) (resid 79 and name HD1*) 4.43 2.62 2.62
assign (resid 19 and name HG12) (resid 19 and name HD1*) 3.96 2.25 2.25
assign (resid 19 and name HG11) (resid 79 and name HD2*) 5.23 3.05 3.05
assign (resid 19 and name HG2*) (resid 19 and name   HA) 4.45 2.63 2.63
assign (resid 19 and name HG2*) (resid 79 and name HD1*) 5.06 3.19 3.20
assign (resid 19 and name HD1*) (resid 15 and name   HA) 4.64 2.73 2.74
assign (resid 19 and name HD1*) (resid 19 and name   HB) 4.81 2.82 2.83
assign (resid 19 and name   HA) (resid 20 and name   HA) 3.92 2.11 2.12
assign (resid 19 and name   HA) (resid 79 and name HD1*) 4.52 2.67 2.67
assign (resid 19 and name   HB) (resid 19 and name HG2*) 4.32 2.56 2.56
assign (resid 19 and name   HB) (resid 19 and name HG12) 3.71 2.00 2.00
assign (resid 20 and name   HA) (resid 23 and name  HD1) 4.12 2.23 2.22
assign (resid 20 and name   HA) (resid 20 and name  HB*) 3.94 2.24 2.23
assign (resid 22 and name   HA) (resid 22 and name  HB2) 4.56 2.46 2.46
assign (resid 22 and name   HA) (resid 25 and name  HB*) 4.83 2.83 2.83
assign (resid 23 and name  HD2) (resid 23 and name  HE1) 4.50 2.43 2.43
assign (resid 23 and name  HD1) (resid 19 and name HG2*) 4.97 2.91 2.92
assign (resid 23 and name  HD2) (resid 23 and name  HD1) 3.92 2.22 2.22
assign (resid 23 and name  HD2) (resid 19 and name HG2*) 4.66 2.74 2.74
assign (resid 23 and name  HE1) (resid 23 and name  HD1) 4.32 2.33 2.33
assign (resid 24 and name  HB*) (resid 24 and name   HA) 3.75 2.04 2.04
assign (resid 25 and name   HA) (resid 28 and name  HB2) 4.35 2.35 2.34
assign (resid 25 and name   HA) (resid 25 and name  HB*) 4.10 2.40 2.41
assign (resid 26 and name   HA) (resid 29 and name  HB2) 4.22 2.27 2.28
assign (resid 27 and name  HA) (resid 27 and name  HB1) 4.65 2.51 2.50
assign (resid 27 and name  HA) (resid 27 and name  HB2) 4.54 2.45 2.45
assign (resid 27 and name  HA) (resid 30 and name  HB1) 4.62 2.62 2.62
assign (resid 28 and name  HG2) (resid 28 and name  HA) 4.42 2.38 2.38
assign (resid 28 and name  HG1) (resid 28 and name  HB1) 4.10 2.21 2.21
assign (resid 28 and name  HG2) (resid 28 and name  HG1) 3.54 1.84 1.83
assign (resid 28 and name  HG2) (resid 28 and name  HB1) 3.77 2.04 2.03
assign (resid 28 and name  HA) (resid 28 and name  HG1) 4.62 2.49 2.49
assign (resid 29 and name  HB2) (resid 29 and name  HA) 4.49 2.42 2.42
assign (resid 29 and name  HB2) (resid 29 and name  HB1) 3.83 2.13 2.13
assign (resid 30 and name  HA) (resid 30 and name  HB2) 4.46 2.40 2.41
assign (resid 30 and name  HB2) (resid 30 and name  HB1) 4.11 2.41 2.41
assign (resid 31 and name  HB*) (resid 32 and name  HB2) 4.71 2.77 2.77
assign (resid 31 and name  HB*) (resid 30 and name  HA) 4.62 2.72 2.71
assign (resid 31 and name  HB*) (resid 32 and name  HA) 4.56 2.69 2.70
assign (resid 31 and name  HB*) (resid 44 and name  HB) 4.69 2.76 2.76
assign (resid 31 and name  HB*) (resid 41 and name  HA) 4.85 2.85 2.84
assign (resid 31 and name  HB*) (resid 31 and name  HA) 4.32 2.56 2.55
assign (resid 32 and name  HA) (resid 32 and name  HB1) 3.93 2.12 2.12
assign (resid 32 and name  HA) (resid 32 and name  HB2) 3.87 2.09 2.09
assign (resid 32 and name  HB2) (resid 32 and name  HB1) 3.69 1.99 1.98
assign (resid 34 and name  HA2) (resid 34 and name  HA1) 3.82 2.06 2.06
assign (resid 35 and name  HA1) (resid 35 and name  HA2) 3.81 2.12 2.11
assign (resid 36 and name  HA) (resid 36 and name  HB1) 4.51 2.43 2.43
assign (resid 36 and name  HA) (resid 36 and name  HB2) 4.61 2.49 2.48
assign (resid 36 and name  HB1) (resid 74 and name  HA) 3.56 1.86 1.85
assign (resid 36 and name  HB1) (resid 72 and name  HB) 4.04 2.18 2.19
assign (resid 36 and name  HB1) (resid 36 and name  HB2) 4.02 2.32 2.32
assign (resid 36 and name  HB1) (resid 72 and name HG2*) 4.95 2.90 2.89
assign (resid 37 and name HG2*) (resid 37 and name  HB) 4.80 2.82 2.82
assign (resid 37 and name HG2*) (resid 37 and name  HA) 4.21 2.50 2.50
assign (resid 37 and name HG2*) (resid 30 and name  HA) 4.45 2.62 2.62
assign (resid 37 and name HG2*) (resid 42 and name  HA) 4.71 2.77 2.77
assign (resid 37 and name HG2*) (resid 42 and name  HB1) 4.38 2.59 2.59
assign (resid 37 and name HG2*) (resid 37 and name HD1*) 4.45 2.75 2.75
assign (resid 37 and name HD1*) (resid 37 and name  HA) 4.91 2.88 2.88
assign (resid 37 and name HD1*) (resid 73 and name HG2*) 4.71 3.00 3.00
assign (resid 37 and name HD1*) (resid 37 and name  HB) 4.71 2.77 2.77
assign (resid 37 and name HD1*) (resid 73 and name  HB) 5.00 2.93 2.92
assign (resid 37 and name HD1*) (resid 29 and name  HB2) 5.00 2.92 2.92
assign (resid 37 and name HD1*) (resid 29 and name  HB1) 4.83 2.83 2.83
assign (resid 37 and name HD1*) (resid 26 and name  HA) 5.07 2.96 2.96
assign (resid 37 and name  HB) (resid 73 and name  HB) 4.31 2.56 2.57
assign (resid 38 and name  HA) (resid 38 and name  HB1) 3.93 2.12 2.12
assign (resid 38 and name  HA) (resid 38 and name  HB2) 3.82 2.06 2.06
assign (resid 38 and name  HA) (resid 72 and name HG2*) 4.17 2.47 2.47
assign (resid 38 and name  HA) (resid 73 and name HG12) 4.61 2.49 2.48
assign (resid 38 and name  HB1) (resid 38 and name  HB2) 3.60 1.90 1.90
assign (resid 39 and name HG2*) (resid 59 and name  HA) 5.12 3.00 2.99
assign (resid 39 and name HG2*) (resid 39 and name  HB) 3.90 2.20 2.19
assign (resid 39 and name HG2*) (resid 39 and name  HA) 4.09 2.39 2.39
assign (resid 39 and name HG2*) (resid 59 and name  HB1) 4.47 2.64 2.65
assign (resid 39 and name HG2*) (resid 62 and name  HB) 4.91 2.88 2.88
assign (resid 39 and name HG2*) (resid 62 and name HG11) 4.36 2.58 2.58
assign (resid 39 and name HG2*) (resid 62 and name HD1*) 4.38 2.68 2.69
assign (resid 39 and name HG2*) (resid 62 and name HG12) 3.70 2.00 2.00
assign (resid 39 and name  HB) (resid 58 and name HD2*) 5.17 3.02 3.01
assign (resid 39 and name  HB) (resid 39 and name  HA) 3.81 2.06 2.06
assign (resid 39 and name  HA) (resid 62 and name HG12) 3.71 2.00 2.00
assign (resid 39 and name  HA) (resid 42 and name HD2*) 4.95 2.90 2.90
assign (resid 40 and name  HG1) (resid 40 and name  HA) 4.05 2.18 2.19
assign (resid 40 and name  HG2) (resid 40 and name  HA) 4.54 2.45 2.45
assign (resid 40 and name  HG1) (resid 41 and name   HA) 4.41 2.38 2.38
assign (resid 40 and name  HG1) (resid 40 and name  HB1) 3.73 2.01 2.01
assign (resid 40 and name  HG1) (resid 40 and name  HB2) 3.60 1.90 1.90
assign (resid 40 and name  HG2) (resid 40 and name  HB2) 3.70 2.00 2.00
assign (resid 40 and name  HB1) (resid 40 and name   HA) 3.75 2.02 2.03
assign (resid 40 and name  HB1) (resid 39 and name   HB) 4.23 2.28 2.29
assign (resid 40 and name  HB2) (resid 40 and name   HA) 4.20 2.27 2.26
assign (resid 40 and name   HA) (resid 58 and name HD2*) 4.97 2.91 2.92
assign (resid 42 and name HD2*) (resid 42 and name  HB2) 4.76 2.80 2.80
assign (resid 42 and name HD2*) (resid 42 and name   HG) 4.62 2.73 2.72
assign (resid 42 and name HD2*) (resid 42 and name  HB1) 4.33 2.57 2.57
assign (resid 42 and name HD1*) (resid 42 and name   HA) 4.09 2.39 2.39
assign (resid 42 and name HD1*) (resid 42 and name  HB1) 4.11 2.41 2.41
assign (resid 42 and name HD1*) (resid 42 and name   HG) 4.12 2.42 2.42
assign (resid 42 and name HD1*) (resid 42 and name  HB2) 4.58 2.70 2.70
assign (resid 42 and name  HB2) (resid 42 and name  HB1) 3.72 2.01 2.01
assign (resid 42 and name  HB2) (resid 42 and name   HA) 4.35 2.35 2.34
assign (resid 42 and name  HB1) (resid 42 and name   HA) 4.61 2.49 2.48
assign (resid 42 and name  HB1) (resid 42 and name   HG) 4.08 2.20 2.20
assign (resid 42 and name   HA) (resid 42 and name   HG) 4.36 2.35 2.34
assign (resid 43 and name  HA2) (resid 43 and name  HA1) 3.81 2.11 2.11
assign (resid 43 and name  HA2) (resid 58 and name HD1*) 4.90 3.10 3.10
assign (resid 44 and name HG2*) (resid 44 and name   HB) 4.10 2.40 2.40
assign (resid 44 and name HG2*) (resid 45 and name   HA) 4.86 2.85 2.85
assign (resid 44 and name HG2*) (resid 44 and name   HA) 4.09 2.39 2.40
assign (resid 44 and name   HB) (resid 41 and neme   HA) 4.17 2.25 2.25
assign (resid 44 and name   HB) (resid 44 and name   HA) 4.28 2.31 2.31
assign (resid 44 and name   HA) (resid 47 and name  HB2) 4.50 2.43 2.43
assign (resid 44 and name   HA) (resid 47 and name  HB1) 4.28 2.31 2.30
assign (resid 45 and name HG1*) (resid 29 and name   HA) 5.01 2.93 2.93
assign (resid 45 and name HG1*) (resid 45 and name   HB) 4.23 2.51 2.50
assign (resid 45 and name HG1*) (resid 45 and name   HA) 4.33 2.56 2.57
assign (resid 45 and name HG1*) (resid 45 and name HG2*) 4.30 2.60 2.60
assign (resid 45 and name HG2*) (resid 45 and name   HA) 4.37 2.58 2.58
assign (resid 45 and name HG2*) (resid 45 and name   HB) 4.11 2.41 2.41
assign (resid 45 and name   HA) (resid 48 and name  HB1) 4.58 2.47 2.46
assign (resid 45 and name   HA) (resid 45 and name   HB) 4.56 2.46 2.46
assign (resid 45 and name   HB) (resid 42 and name   HA) 3.86 2.08 2.08
assign (resid 45 and name   HB) (resid 46 and name   HA) 4.62 2.49 2.49
assign (resid 46 and name  HG2) (resid 46 and name   HA) 4.50 2.43 2.43
assign (resid 46 and name  HG1) (resid 46 and name  HG2) 3.52 1.82 1.81
assign (resid 46 and name  HG1) (resid 46 and name  HB2) 4.67 2.52 2.52
assign (resid 46 and name  HG1) (resid 46 and name  HE*) 5.08 2.97 2.97
assign (resid 46 and name  HG1) (resid 46 and name  HB1) 4.46 2.40 2.41
assign (resid 46 and name  HG2) (resid 46 and name  HB1) 4.23 2.28 2.28
assign (resid 46 and name  HG2) (resid 46 and name  HE*) 5.12 2.99 2.99
assign (resid 46 and name   HA) (resid 46 and name  HB2) 4.04 2.18 2.18
assign (resid 46 and name  HB2) (resid 46 and name  HB1) 4.10 2.40 2.40
assign (resid 47 and name  HG1) (resid 53 and name  HD1) 4.50 2.42 2.42
assign (resid 47 and name  HG1) (resid 47 and name   HA) 4.25 2.45 2.45
assign (resid 47 and name  HG2) (resid 47 and name   HA) 4.33 2.33 2.33
assign (resid 47 and name  HG2) (resid 53 and name  HD2) 4.04 2.18 2.19
assign (resid 47 and name  HG1) (resid 53 and name  HD2) 3.92 2.11 2.12
assign (resid 47 and name   HA) (resid 47 and name  HB1) 4.20 2.27 2.26
assign (resid 47 and name  HB1) (resid 53 and name  HD2) 4.00 2.16 2.16
assign (resid 48 and name  HG2) (resid 48 and name   HA) 4.52 2.72 2.72
assign (resid 48 and name  HG1) (resid 48 and name  HG2) 3.28 1.58 1.58
assign (resid 48 and name  HB2) (resid 48 and name  HG2) 4.33 2.33 2.33
assign (resid 48 and name  HB2) (resid 48 and name   HA) 4.38 2.35 2.36
assign (resid 48 and name  HB1) (resid 48 and name   HA) 4.55 2.45 2.46
assign (resid 48 and name  HG1) (resid 48 and name  HB1) 4.36 2.35 2.35
assign (resid 48 and name  HG1) (resid 48 and name  HB2) 4.43 2.39 2.39
assign (resid  48 and name  HG2) (resid  48 and name  HE*)  4.73  2.78  2.79
assign (resid  48 and name   HA) (resid  48 and name  HG1)  4.44  2.39  2.39
assign (resid  49 and name   HA) (resid  49 and name  HB1)  3.71  2.00  2.00
assign (resid  49 and name   HA) (resid  49 and name  HB2)  3.67  1.97  1.97
assign (resid  49 and name   HA) (resid  49 and name HD2*)  5.12  2.99  2.98
assign (resid  49 and name  HB1) (resid  49 and name HD2*)  5.02  2.94  2.94
assign (resid  50 and name  HA2) (resid  50 and name  HA1)  3.74  2.02  2.02
assign (resid  51 and name   HA) (resid  52 and name  HB1)  4.62  2.50  2.50
assign (resid  51 and name   HA) (resid  52 and name  HB2)  4.23  2.28  2.29
assign (resid  51 and name   HA) (resid  51 and name  HB1)  4.16  2.25  2.25
assign (resid  51 and name   HA) (resid  47 and name  HG1)  4.54  2.45  2.45
assign (resid  51 and name   HA) (resid  51 and name  HB2)  4.58  2.47  2.46
assign (resid  51 and name  HB2) (resid  51 and name  HB1)  3.88  2.18  2.18
assign (resid  52 and name   HA) (resid  53 and name  HD1)  3.77  2.04  2.03
assign (resid  52 and name   HA) (resid  53 and name  HD2)  4.06  2.19  2.20
assign (resid  52 and name   HA) (resid  52 and name  HB1)  4.12  2.23  2.22
assign (resid  52 and name   HA) (resid  52 and name  HB2)  4.00  2.16  2.16
assign (resid  52 and name  HB1) (resid  52 and name  HB2)  3.50  1.80  1.80
assign (resid  53 and name  HD1) (resid  53 and name  HD2)  3.60  1.90  1.90
assign (resid  53 and name  HD1) (resid  47 and name  HB1)  4.19  2.26  2.25
assign (resid  53 and name  HD1) (resid  46 and name  HB1)  4.23  2.28  2.28
assign (resid  53 and name  HD1) (resid  53 and name  HB2)  4.53  2.44  2.44
assign (resid  53 and name   HA) (resid  53 and name  HB1)  4.13  2.22  2.23
assign (resid  53 and name   HA) (resid  53 and name  HB2)  4.10  2.21  2.21
assign (resid  54 and name HG2*) (resid  54 and name   HB)  3.79  2.09  2.09
assign (resid  54 and name HG2*) (resid  54 and name   HA)  3.91  2.21  2.21
assign (resid  54 and name   HB) (resid  54 and name   HA)  3.47  1.77  1.77
assign (resid  54 and name   HB) (resid  53 and name   HA)  4.14  2.23  2.23
assign (resid  55 and name  HB2) (resid  56 and name   HA)  4.11  2.22  2.21
assign (resid  55 and name  HB2) (resid  55 and name   HA)  4.35  2.35  2.34
assign (resid  55 and name  HB1) (resid  55 and name   HA)  3.95  2.13  2.13
assign (resid  56 and name  HG2) (resid  56 and name   HA)  4.47  2.41  2.41
assign (resid  56 and name  HG2) (resid  56 and name  HB1)  4.12  2.22  2.22
assign (resid  56 and name  HG1) (resid  56 and name   HA)  4.14  2.23  2.23
assign (resid  56 and name  HG1) (resid  56 and name  HB1)  4.23  2.28  2.29
assign (resid  56 and name   HA) (resid  59 and name  HB1)  4.60  2.48  2.47
assign (resid  58 and name   HG) (resid  53 and name  HB2)  4.11  2.22  2.21
assign (resid  58 and name   HG) (resid  61 and name   HB)  4.02  2.17  2.17
assign (resid  58 and name   HG) (resid  58 and name HD2*)  4.43  2.62  2.62
assign (resid  58 and name   HG) (resid  58 and name HD1*)  4.47  2.64  2.65
assign (resid  58 and name HD2*) (resid  43 and name  HA2)  5.00  2.93  2.92
assign (resid  58 and name HD2*) (resid  58 and name   HA)  3.98  2.29  2.28
assign (resid  58 and name HD2*) (resid  53 and name  HB1)  5.00  2.93  2.92
assign (resid  58 and name   HA) (resid  58 and name   HG)  4.07  2.19  2.20
assign (resid  59 and name   HA) (resid  59 and name  HB1)  4.46  2.41  2.41
assign (resid  59 and name   HA) (resid  59 and name  HB2)  4.19  2.26  2.25
assign (resid  59 and name   HA) (resid  62 and name   HB)  4.14  2.23  2.24
assign (resid  59 and name   HA) (resid  62 and name HG2*)  4.76  2.80  2.80
assign (resid  59 and name  HB2) (resid  60 and name   HA)  4.38  2.36  2.37
assign (resid  60 and name   HA) (resid  63 and name  HB1)  4.43  2.39  2.39
assign (resid  60 and name   HA) (resid  63 and name  HB2)  4.19  2.26  2.26
assign (resid  60 and name   HA) (resid  60 and name  HB*)  3.88  2.18  2.18
assign (resid  60 and name  HB*) (resid  57 and name   HA)  4.30  2.55  2.55
assign (resid  61 and name HG2*) (resid  61 and name   HA)  4.12  2.42  2.42
assign (resid  61 and name HD1*) (resid  61 and name HG2*)  4.52  2.82  2.82
assign (resid  61 and name HD1*) (resid  61 and name   HA)  4.96  2.90  2.91
assign (resid  61 and name HD1*) (resid  61 and name   HB)  4.43  2.62  2.62
assign (resid  61 and name HD1*) (resid  61 and name HG11)  4.12  2.42  2.42
assign (resid  61 and name HD1*) (resid  61 and name HG12)  3.77  2.07  2.06
assign (resid  61 and name   HB) (resid  61 and name HG2*)  4.06  2.36  2.36
assign (resid  61 and name   HA) (resid  61 and name   HB)  4.57  2.47  2.46
assign (resid  61 and name HG11) (resid  61 and name HG12)  3.81  2.06  2.06
assign (resid 62 and name HG12) (resid 62 and name HG11) 3.85 2.15 2.15
assign (resid 62 and name HG12) (resid 42 and name HD2*) 4.92 2.88 2.88
assign (resid 62 and name HG12) (resid 62 and name HD1*) 4.61 2.72 2.71
assign (resid 62 and name HG2*) (resid 62 and name   HA) 4.18 2.48 2.48
assign (resid 62 and name HG2*) (resid 62 and name   HB) 3.97 2.27 2.27
assign (resid 62 and name HG2*) (resid 62 and name HG11) 4.71 2.77 2.77
assign (resid 62 and name HD1*) (resid 72 and name   HA) 4.86 2.85 2.84
assign (resid 62 and name HD1*) (resid 62 and name   HA) 4.62 2.72 2.72
assign (resid 62 and name HD1*) (resid 39 and name   HA) 4.53 2.67 2.67
assign (resid 62 and name HD1*) (resid 62 and name   HB) 4.57 2.69 2.70
assign (resid 62 and name HD1*) (resid 62 and name HG11) 4.54 2.68 2.69
assign (resid 62 and name   HA) (resid 62 and name HG12) 4.61 2.49 2.48
assign (resid 62 and name   HA) (resid 65 and name HG1*) 4.99 2.92 2.92
assign (resid 62 and name   HA) (resid 62 and name   HB) 4.65 2.51 2.51
assign (resid 62 and name   HB) (resid 62 and name HG11) 4.58 2.47 2.47
assign (resid 62 and name   HA) (resid 73 and name HD1*) 4.90 2.87 2.87
assign (resid 62 and name HG11) (resid 62 and name   HA) 4.64 2.50 2.50
assign (resid 63 and name   HA) (resid 63 and name  HG2) 3.87 2.09 2.09
assign (resid 63 and name   HA) (resid 63 and name  HB1) 3.84 2.07 2.07
assign (resid 63 and name  HB2) (resid 63 and name   HA) 3.69 1.99 1.99
assign (resid 64 and name  HB1) (resid 64 and name   HA) 4.33 2.33 2.33
assign (resid 65 and name   HA) (resid 66 and name   HA) 4.23 2.28 2.28
assign (resid 65 and name   HA) (resid 64 and name   HA) 4.22 2.28 2.28
assign (resid 65 and name   HA) (resid 65 and name   HB) 4.23 2.28 2.29
assign (resid 65 and name   HA) (resid 65 and name HG2*) 4.93 2.89 2.89
assign (resid 65 and name   HA) (resid 65 and name HG1*) 4.42 2.61 2.61
assign (resid 65 and name HG2*) (resid 65 and name   HB) 4.20 2.50 2.50
assign (resid 65 and name HG2*) (resid 81 and name  HG2) 4.95 2.90 2.89
assign (resid 65 and name HG2*) (resid 81 and name  HE*) 5.53 3.44 3.44
assign (resid 65 and name HG1*) (resid 66 and name   HA) 4.67 2.75 2.75
assign (resid 65 and name HG1*) (resid 81 and name  HE*) 5.13 3.22 3.22
assign (resid 65 and name HG1*) (resid 73 and name HG11) 4.16 2.46 2.46
assign (resid 65 and name HG1*) (resid 65 and name HG2*) 4.13 2.43 2.44
assign (resid 65 and name HG1*) (resid 65 and name   HB) 4.08 2.38 2.38
assign (resid 65 and name   HB) (resid 66 and name   HA) 4.50 2.42 2.42
assign (resid 65 and name   HB) (resid 81 and name  HE*) 4.75 2.79 2.79
assign (resid 66 and name   HA) (resid 67 and name  HB2) 4.75 2.56 2.56
assign (resid 67 and name   HA) (resid 67 and name  HB1) 3.89 2.10 2.10
assign (resid 68 and name   HA) (resid 68 and name  HB1) 4.06 2.19 2.20
assign (resid 68 and name   HA) (resid 68 and name  HB2) 4.14 2.23 2.23
assign (resid 69 and name  HA1) (resid 69 and name  HA2) 3.74 2.02 2.02
assign (resid 70 and name  HB1) (resid 70 and name  HB2) 3.93 2.23 2.23
assign (resid 71 and name  HA2) (resid 71 and name  HA1) 3.98 2.28 2.28
assign (resid 72 and name HG2*) (resid 72 and name   HA) 3.88 2.18 2.18
assign (resid 72 and name HG2*) (resid 38 and name  HB1) 4.45 2.62 2.62
assign (resid 72 and name HG2*) (resid 38 and name  HB2) 5.06 2.96 2.96
assign (resid 72 and name HG2*) (resid 72 and name   HB) 3.89 2.19 2.19
assign (resid 72 and name   HB) (resid 36 and name  HB2) 4.60 2.48 2.48
assign (resid 72 and name   HB) (resid 72 and name   HA) 3.90 2.10 2.10
assign (resid 73 and name HG12) (resid 73 and name HG11) 4.05 2.35 2.35
assign (resid 73 and name HG11) (resid 73 and name HG2*) 5.00 2.93 2.92
assign (resid 73 and name HG12) (resid 73 and name HG2*) 5.08 2.97 2.97
assign (resid 73 and name HG11) (resid 73 and name HD1*) 4.01 2.31 2.31
assign (resid 73 and name HG11) (resid 62 and name HD1*) 4.95 2.90 2.89
assign (resid 73 and name HG2*) (resid 73 and name   HA) 5.00 2.93 2.93
assign (resid 73 and name HG2*) (resid 78 and name   HA) 4.64 2.73 2.74
assign (resid 73 and name HG2*) (resid 73 and name HD1*) 5.13 3.22 3.22
assign (resid 73 and name HD1*) (resid 62 and name HG12) 4.86 2.85 2.85
assign (resid 73 and name HD1*) (resid 62 and name HD1*) 4.52 2.82 2.83
assign (resid 73 and name   HB) (resid 73 and name HG2*) 4.93 2.89 2.89
assign (resid 73 and name   HB) (resid 73 and name HG12) 4.65 2.51 2.51
assign (resid 73 and name   HA) (resid 73 and name HG12) 4.65 2.51 2.50
assign (resid 73 and name  HA) (resid 73 and name HD1*) 5.01 2.93 2.93
assign (resid 74 and name  HA) (resid 36 and name  HA) 4.23 2.43 2.42
assign (resid 74 and name  HA) (resid 74 and name  HB2) 4.60 2.48 2.48
assign (resid 74 and name  HA) (resid 74 and name  HB1) 4.45 2.40 2.40
assign (resid 74 and name HB2) (resid 74 and name  HB1) 4.09 2.39 2.39
assign (resid 74 and name HB2) (resid 75 and name  HB1) 3.82 2.06 2.06
assign (resid 75 and name HB2) (resid 75 and name  HB1) 3.98 2.29 2.28
assign (resid 78 and name HB2) (resid 78 and name  HB1) 4.16 2.46 2.46
assign (resid 78 and name  HA) (resid 81 and name  HB1) 4.33 2.53 2.52
assign (resid 78 and name  HA) (resid 78 and name  HB2) 4.74 2.56 2.56
assign (resid 79 and name  HG) (resid 79 and name HD1*) 4.33 2.57 2.56
assign (resid 79 and name  HG) (resid 79 and name HD2*) 4.79 2.81 2.82
assign (resid 79 and name HD2*) (resid 14 and name HD1*) 4.88 3.09 3.10
assign (resid 79 and name HD2*) (resid 19 and name HG12) 4.63 2.72 2.73
assign (resid 79 and name HD2*) (resid 79 and name  HA) 5.21 3.04 3.04
assign (resid 79 and name HD2*) (resid 79 and name  HB1) 4.49 2.65 2.64
assign (resid 79 and name HD2*) (resid 79 and name  HB2) 4.19 2.49 2.49
assign (resid 79 and name HD1*) (resid 79 and name  HA) 4.26 2.53 2.53
assign (resid 79 and name  HA) (resid 79 and name  HB2) 4.50 2.43 2.43
assign (resid 79 and name  HA) (resid 79 and name  HG) 4.56 2.46 2.46
assign (resid 80 and name HG1*) (resid 80 and name  HA) 5.21 3.04 3.04
assign (resid 80 and name HG1*) (resid  7 and name  HA) 4.33 2.57 2.57
assign (resid 80 and name HG1*) (resid 77 and name  HA) 3.98 2.28 2.27
assign (resid 80 and name HG1*) (resid 80 and name  HB) 4.21 2.50 2.50
assign (resid 80 and name HG2*) (resid  6 and name  HG2) 4.83 2.83 2.83
assign (resid 80 and name HG2*) (resid 80 and name  HA) 4.49 2.65 2.65
assign (resid 80 and name  HA) (resid 83 and name  HB) 4.46 2.66 2.66
assign (resid 80 and name  HA) (resid 83 and name HG1*) 4.29 2.54 2.54
assign (resid 80 and name  HB) (resid 77 and name  HA) 4.74 2.56 2.55
assign (resid 80 and name  HB) (resid 80 and name HG2*) 4.50 2.66 2.66
assign (resid 81 and name  HA) (resid 81 and name  HB2) 4.31 2.32 2.33
assign (resid 81 and name  HA) (resid 81 and name  HB1) 4.34 2.34 2.34
assign (resid 81 and name  HA) (resid 80 and name  HB) 4.41 2.38 2.38
assign (resid 82 and name  HA) (resid 82 and name  HB1) 4.23 2.28 2.28
assign (resid 82 and name  HA) (resid 82 and name  HB2) 4.50 2.43 2.42
assign (resid 82 and name HB1) (resid 82 and name  HB2) 4.08 2.38 2.38
assign (resid 83 and name HG1*) (resid 10 and name  HB*) 5.32 3.33 3.33
assign (resid 83 and name HG1*) (resid 10 and name  HA) 5.15 3.01 3.00
assign (resid 83 and name HG1*) (resid 83 and name  HA) 4.21 2.50 2.50
assign (resid 83 and name HG1*) (resid 83 and name  HB) 4.21 2.50 2.50
assign (resid 83 and name HG2*) (resid 13 and name  HB2) 4.91 2.88 2.88
assign (resid 83 and name HG2*) (resid 83 and name  HA) 4.45 2.62 2.62
assign (resid 83 and name HG2*) (resid 83 and name  HB) 4.08 2.38 2.38
assign (resid 83 and name HG2*) (resid 83 and name HG1*) 4.34 2.64 2.65
assign (resid 84 and name HG2) (resid 80 and name  HA) 4.15 2.24 2.24
assign (resid 84 and name HG2) (resid 84 and name  HG1) 3.52 1.82 1.82
assign (resid 84 and name HG2) (resid 83 and name HG1*) 4.28 2.54 2.54
assign (resid 84 and name  HA) (resid 84 and name  HG2) 4.12 2.22 2.22
assign (resid 85 and name  HA) (resid 85 and name  HG2) 4.03 2.17 2.17
assign (resid 86 and name HG2) (resid 86 and name  HA) 4.40 2.37 2.37
assign (resid 86 and name HB2) (resid 86 and name  HB1) 3.40 1.70 1.69
assign (resid 87 and name HG1) (resid 87 and name  HB2) 4.25 2.29 2.29
assign (resid 87 and name  HA) (resid 87 and name  HB2) 4.47 2.41 2.42
assign (resid 88 and name  HA) (resid 89 and name  HB2) 4.54 2.45 2.45
assign (resid 89 and name  HA) (resid 88 and name  HA) 4.57 2.47 2.46
assign (resid 89 and name  HA) (resid 89 and name  HB1) 3.50 1.80 1.80
assign (resid 89 and name HB2) (resid 89 and name  HA) 3.90 2.10 2.10
assign (resid 90 and name  HA) (resid 90 and name  HB*) 4.02 2.32 2.33
assign (resid 91 and name  HA) (resid 91 and name  HB2) 3.91 2.11 2.11
assign (resid 91 and name HB1) (resid 91 and name  HA) 4.16 2.25 2.25
assign (resid 91 and name HB1) (resid 91 and name  HG1) 3.71 2.00 2.00
assign (resid 93 and name  HA) (resid 93 and name  HB1) 4.25 2.29 2.29
assign (resid  93 and name   HA) (resid  93 and name  HB2) 4.23 2.28 2.29
assign (resid  93 and name   HA) (resid  93 and name  HG1) 4.52 2.44 2.44
assign (resid  93 and name   HA) (resid  93 and name  HD2) 4.55 2.45 2.45
assign (resid  93 and name  HD1) (resid  93 and name   HA) 4.65 2.51 2.51
assign (resid  93 and name  HD1) (resid  93 and name  HB1) 3.83 2.06 2.07
assign (resid  93 and name  HD1) (resid  93 and name  HB2) 3.53 1.83 1.83
assign (resid  94 and name  HB2) (resid  94 and name   HA) 3.81 2.05 2.06
assign (resid  94 and name  HB1) (resid  94 and name   HA) 3.85 2.08 2.07
assign (resid  94 and name  HB2) (resid  94 and name  HB1) 3.21 1.51 1.51
assign (resid  95 and name   HA) (resid  98 and name  HB1) 3.82 2.06 2.06
assign (resid  95 and name   HA) (resid  98 and name HD1*) 4.82 2.83 2.83
assign (resid  97 and name   HA) (resid 100 and name  HB1) 4.37 2.36 2.36
assign (resid  97 and name   HA) (resid 100 and name  HB2) 4.69 2.53 2.52
assign (resid  97 and name   HA) (resid  97 and name  HB1) 3.98 2.15 2.15
assign (resid  98 and name   HG) (resid  98 and name HD1*) 4.31 2.55 2.55
assign (resid  98 and name   HG) (resid  98 and name HD2*) 4.34 2.57 2.57
assign (resid  98 and name   HA) (resid  98 and name  HB2) 4.12 2.23 2.22
assign (resid  98 and name   HA) (resid  98 and name  HB1) 3.95 2.12 2.12
assign (resid  99 and name   HA) (resid 102 and name  HB1) 4.47 2.67 2.67
assign (resid  99 and name   HA) (resid 102 and name  HB2) 4.47 2.41 2.42
assign (resid  99 and name   HA) (resid  99 and name  HB*) 3.98 2.28 2.28
assign (resid  99 and name  HB*) (resid  96 and name   HA) 4.79 2.82 2.81
assign (resid 100 and name   HA) (resid 100 and name  HB1) 3.91 2.11 2.11
assign (resid 100 and name   HA) (resid 100 and name  HB2) 4.04 2.17 2.17
assign (resid 100 and name   HA) (resid 103 and name  HG1) 4.22 2.27 2.28
assign (resid 100 and name   HA) (resid  99 and name  HB*) 4.83 2.83 2.83
assign (resid 100 and name  HB2) (resid 100 and name  HB1) 3.29 1.59 1.58
assign (resid 101 and name  HB2) (resid 101 and name  HB1) 3.54 1.84 1.84
assign (resid 101 and name  HB2) (resid  98 and name   HA) 4.47 2.41 2.41
assign (resid 101 and name  HB1) (resid  98 and name   HA) 4.50 2.43 2.43
assign (resid 101 and name  HB2) (resid 101 and name   HA) 4.46 2.40 2.41
assign (resid 101 and name  HB1) (resid 101 and name   HA) 4.47 2.41 2.41
assign (resid 101 and name   HA) (resid 104 and name   HB) 4.07 2.19 2.20
assign (resid 102 and name   HA) (resid 102 and name  HB1) 4.23 2.28 2.29
assign (resid 102 and name   HA) (resid 102 and name  HB2) 4.31 2.32 2.33
assign (resid 103 and name  HG2) (resid 103 and name   HA) 4.62 2.50 2.50
assign (resid 103 and name  HG1) (resid 103 and name   HA) 4.12 2.23 2.22
assign (resid 104 and name HG11) (resid 100 and name  HB1) 3.83 2.06 2.07
assign (resid 104 and name HG12) (resid 104 and name   HB) 4.50 2.43 2.42
assign (resid 104 and name HG12) (resid 104 and name HG11) 3.71 2.00 2.00
assign (resid 104 and name HG11) (resid 104 and name HD1*) 4.51 2.66 2.66
assign (resid 104 and name HG12) (resid 104 and name HD1*) 4.53 2.67 2.67
assign (resid 104 and name HG12) (resid 104 and name HG2*) 4.73 2.78 2.78
assign (resid 104 and name HG11) (resid 104 and name HG2*) 5.19 3.03 3.03
assign (resid 104 and name HG11) (resid 104 and name   HA) 4.67 2.52 2.51
assign (resid 104 and name HG12) (resid 104 and name   HA) 4.60 2.48 2.48
assign (resid 104 and name HG2*) (resid 104 and name   HA) 4.12 2.42 2.42
assign (resid 104 and name HG2*) (resid 104 and name   HB) 3.98 2.29 2.28
assign (resid 104 and name HD1*) (resid 104 and name   HA) 4.69 2.76 2.76
assign (resid 104 and name HD1*) (resid 104 and name   HB) 4.52 2.67 2.67
assign (resid 104 and name   HB) (resid 104 and name   HA) 4.62 2.49 2.49
assign (resid 104 and name   HB) (resid 104 and name HG11) 4.35 2.35 2.34
assign (resid 104 and name HG11) (resid 100 and name  HB2) 4.22 2.27 2.28
assign (resid 107 and name  HG2) (resid 105 and name   HA) 4.55 2.45 2.45
assign (resid 107 and name  HG1) (resid 107 and name   HA) 4.71 2.54 2.54
assign (resid 107 and name  HG2) (resid 107 and name   HA) 4.11 2.22 2.21
assign (resid 107 and name  HG2) (resid 117 and name  HG1) 4.71 2.54 2.54
assign (resid 107 and name  HG2) (resid 107 and name  HG1) 3.11 1.41 1.41
assign (resid 108 and name   HA) (resid 108 and name  HB1) 3.73 2.00 2.01
assign (resid 108 and name   HA) (resid 108 and name  HB2) 3.76 2.03 2.03
assign (resid 108 and name  HB2) (resid 108 and name  HB1) 3.67 1.97 1.97
assign (resid 109 and name   HA) (resid 106 and name  HB2) 4.42 2.39 2.38
assign (resid 109 and name   HA) (resid 109 and name  HB*) 3.98 2.28 2.27
assign (resid 110 and name   HA) (resid 110 and name  HB1) 3.83 2.06 2.07
assign (resid 110 and name   HA) (resid 110 and name  HB2) 3.81 2.05 2.06
assign (resid 110 and name  HB2) (resid 110 and name  HB1) 3.84 2.14 2.14
assign (resid 110 and name  HB2) (resid 108 and name  HB1) 3.96 2.13 2.13
assign (resid 111 and name  HA2) (resid 111 and name  HA1) 3.76 2.03 2.03
assign (resid 112 and name   HA) (resid 112 and name  HB1) 4.34 2.34 2.34
assign (resid 112 and name   HA) (resid 112 and name  HB2) 4.44 2.40 2.39
assign (resid 112 and name   HA) (resid 113 and name HG12) 4.63 2.50 2.50
assign (resid 112 and name  HB1) (resid 112 and name  HB2) 3.62 1.93 1.92
assign (resid 113 and name HG11) (resid 113 and name HG12) 3.95 2.25 2.25
assign (resid 113 and name HG11) (resid 113 and name HG2*) 4.98 2.92 2.92
assign (resid 113 and name HG12) (resid 113 and name HD1*) 4.58 2.70 2.70
assign (resid 113 and name HG2*) (resid 113 and name   HA) 4.54 2.68 2.68
assign (resid 113 and name HG2*) (resid 118 and name   HA) 4.70 2.77 2.76
assign (resid 113 and name HG2*) (resid 117 and name  HB1) 4.43 2.62 2.62
assign (resid 113 and name HG2*) (resid 118 and name  HB1) 4.89 2.87 2.87
assign (resid 113 and name HG2*) (resid 113 and name HD1*) 4.30 2.60 2.60
assign (resid 113 and name HG2*) (resid 105 and name  HB2) 4.78 2.81 2.81
assign (resid 113 and name HD1*) (resid 113 and name   HA) 5.03 2.94 2.94
assign (resid 113 and name HD1*) (resid 113 and name   HB) 4.99 2.92 2.92
assign (resid 113 and name HD1*) (resid 149 and name HG2*) 5.57 3.46 3.46
assign (resid 113 and name   HB) (resid 112 and name   HA) 4.32 2.33 2.33
assign (resid 113 and name   HB) (resid 113 and name   HA) 4.45 2.40 2.40
assign (resid 113 and name   HB) (resid 149 and name HG2*) 4.75 2.79 2.79
assign (resid 113 and name   HB) (resid 149 and name   HB) 4.10 2.30 2.30
assign (resid 114 and name   HA) (resid 114 and name  HB1) 4.08 2.20 2.20
assign (resid 114 and name   HA) (resid 114 and name  HB2) 4.05 2.18 2.19
assign (resid 114 and name   HA) (resid 138 and name  HE*) 4.65 2.74 2.74
assign (resid 114 and name  HB2) (resid 114 and name  HB1) 3.98 2.28 2.27
assign (resid 115 and name HG2*) (resid 116 and name   HA) 5.15 3.01 3.01
assign (resid 115 and name HG2*) (resid 115 and name   HA) 4.84 2.84 2.84
assign (resid 115 and name HD1*) (resid 115 and name   HA) 4.57 2.69 2.70
assign (resid 115 and name HD1*) (resid 135 and name  HG1) 5.11 2.99 2.98
assign (resid 115 and name HD1*) (resid 115 and name   HB) 4.13 2.43 2.43
assign (resid 115 and name HD1*) (resid 115 and name HG11) 4.19 2.49 2.49
assign (resid 115 and name HD1*) (resid 115 and name HG12) 4.41 2.61 2.60
assign (resid 115 and name HD1*) (resid 115 and name HG2*) 4.60 2.90 2.90
assign (resid 115 and name   HB) (resid 115 and name   HA) 3.95 2.13 2.13
assign (resid 115 and name   HB) (resid 115 and name HG2*) 4.19 2.49 2.49
assign (resid 115 and name   HB) (resid 115 and name HG12) 4.62 2.49 2.48
assign (resid 115 and name   HB) (resid 115 and name HG11) 4.52 2.44 2.44
assign (resid 115 and name   HA) (resid 138 and name  HE*) 4.21 2.50 2.50
assign (resid 115 and name   HA) (resid 115 and name HG11) 4.12 2.22 2.21
assign (resid 115 and name HG12) (resid 115 and name HG11) 3.50 1.80 1.80
assign (resid 115 and name HG12) (resid 115 and name HG2*) 4.49 2.65 2.64
assign (resid 115 and name HG11) (resid 115 and name HG2*) 4.85 2.85 2.84
assign (resid 116 and name   HA) (resid 116 and name  HB2) 3.65 1.95 1.95
assign (resid 116 and name   HA) (resid 116 and name  HG2) 3.87 2.08 2.08
assign (resid 116 and name   HA) (resid 116 and name  HB1) 3.52 1.82 1.81
assign (resid 116 and name   HA) (resid 116 and name  HG1) 3.90 2.10 2.10
assign (resid 116 and name  HB2) (resid 116 and name  HG1) 3.67 1.97 1.97
assign (resid 117 and name   HA) (resid 117 and name  HB1) 4.10 2.21 2.21
assign (resid 118 and name   HG) (resid 118 and name HD2*) 4.84 2.84 2.84
assign (resid 118 and name   HG) (resid 134 and name HG2*) 5.10 2.98 2.98
assign (resid 118 and name   HG) (resid 118 and name  HB2) 4.58 2.47 2.46
assign (resid 118 and name HD1*) (resid 118 and name   HA) 4.47 2.64 2.65
assign (resid 118 and name HD1*) (resid 118 and name  HB1) 4.02 2.32 2.32
assign (resid 118 and name HD1*) (resid 118 and name   HG) 4.14 2.44 2.45
assign (resid 118 and name HD2*) (resid 118 and name   HA) 4.12 2.42 2.42
assign (resid 118 and name HD2*) (resid 138 and name  HE*) 5.50 3.43 3.43
assign (resid 118 and name HD2*) (resid 137 and name  HB2) 4.91 2.88 2.88
assign (resid 118 and name HD2*) (resid 115 and name HG11) 4.71 2.77 2.77
assign (resid 118 and name HD2*) (resid 118 and name  HB2) 4.69 2.76 2.75
assign (resid 118 and name  HB2) (resid 138 and name  HE*) 4.25 2.52 2.53
assign (resid 118 and name  HB2) (resid 118 and name   HA) 4.21 2.27 2.26
assign (resid 118 and name  HB1) (resid 118 and name   HA) 4.50 2.43 2.42
assign (resid 118 and name  HB2) (resid 118 and name  HB1) 3.59 1.89 1.89
assign (resid 118 and name  HB1) (resid 118 and name HD2*) 4.70 2.77 2.76
assign (resid 118 and name   HA) (resid 121 and name HD1*) 4.76 2.80 2.80
assign (resid 119 and name  HA2) (resid 134 and name HG2*) 4.85 2.85 2.84
assign (resid 119 and name  HA2) (resid 134 and name HD1*) 4.86 2.85 2.84
assign (resid 119 and name  HA1) (resid 134 and name HD1*) 5.02 2.94 2.94
assign (resid 119 and name  HA1) (resid 119 and name  HA2) 3.63 1.93 1.93
assign (resid 120 and name   HA) (resid 120 and name  HB2) 3.84 2.07 2.08
assign (resid 120 and name  HB1) (resid 120 and name   HA) 3.99 2.15 2.15
assign (resid 121 and name HG11) (resid 121 and name   HB) 4.54 2.45 2.45
assign (resid 121 and name HG12) (resid 121 and name HG11) 3.81 2.11 2.11
assign (resid 121 and name HG11) (resid 121 and name HD1*) 4.61 2.72 2.72
assign (resid 121 and name HG11) (resid 121 and name HG2*) 4.32 2.56 2.56
assign (resid 121 and name HG2*) (resid 121 and name   HA) 4.47 2.64 2.64
assign (resid 121 and name HG2*) (resid 122 and name   HA) 5.12 2.99 2.98
assign (resid 121 and name HG2*) (resid 121 and name   HB) 4.25 2.52 2.52
assign (resid 121 and name HD1*) (resid 113 and name HG2*) 5.08 3.20 3.21
assign (resid 121 and name HD1*) (resid 121 and name   HA) 5.06 2.96 2.96
assign (resid 121 and name HD1*) (resid 121 and name   HB) 4.77 2.80 2.80
assign (resid 121 and name   HA) (resid 124 and name  HB*) 4.78 2.81 2.81
assign (resid 121 and name   HA) (resid 121 and name HG12) 3.82 2.06 2.06
assign (resid 122 and name   HG) (resid 122 and name  HB2) 4.30 2.32 2.32
assign (resid 122 and name HD2*) (resid 122 and name   HA) 4.70 2.77 2.76
assign (resid 122 and name HD2*) (resid 125 and name HG2*) 5.00 3.16 3.16
assign (resid 122 and name HD2*) (resid 137 and name HD2*) 4.88 3.09 3.10
assign (resid 122 and name HD2*) (resid 121 and name HG2*) 4.50 2.80 2.80
assign (resid 122 and name HD1*) (resid 122 and name   HA) 4.06 2.36 2.36
assign (resid 122 and name HD1*) (resid 119 and name  HA1) 4.92 2.89 2.88
assign (resid 122 and name HD1*) (resid 134 and name   HA) 4.94 2.90 2.89
assign (resid 122 and name HD1*) (resid 129 and name   HB) 4.33 2.57 2.56
assign (resid 122 and name HD1*) (resid 122 and name   HG) 4.21 2.50 2.50
assign (resid 122 and name HD1*) (resid 122 and name  HB1) 5.06 2.96 2.96
assign (resid 122 and name HD1*) (resid 122 and name  HB2) 4.54 2.68 2.69
assign (resid 122 and name  HB2) (resid 129 and name HG1*) 4.60 2.71 2.71
assign (resid 122 and name   HA) (resid 122 and name  HB2) 4.21 2.27 2.28
assign (resid 122 and name   HA) (resid 125 and name HG2*) 4.62 2.72 2.72
assign (resid 123 and name  HG2) (resid 123 and name   HA) 4.36 2.35 2.35
assign (resid 123 and name  HG1) (resid 123 and name   HA) 4.58 2.47 2.46
assign (resid 123 and name  HG2) (resid 123 and name  HD1) 4.46 2.40 2.41
assign (resid 123 and name  HG2) (resid 123 and name  HD2) 4.61 2.49 2.49
assign (resid 123 and name  HG1) (resid 123 and name  HD2) 3.72 2.01 2.01
assign (resid 123 and name  HG1) (resid 129 and name HG1*) 4.12 2.42 2.42
assign (resid 123 and name  HG1) (resid 134 and name HD1*) 4.47 2.64 2.64
assign (resid 123 and name   HA) (resid 129 and name HG1*) 5.08 2.97 2.97
assign (resid 123 and name  HG1) (resid 123 and name  HD1) 4.42 2.39 2.38
assign (resid 123 and name  HG2) (resid 134 and name HD1*) 4.97 2.91 2.91
assign (resid 124 and name   HA) (resid 124 and name  HB*) 3.89 2.19 2.19
assign (resid 125 and name HG2*) (resid 125 and name   HB) 3.81 2.11 2.10
assign (resid 125 and name HG2*) (resid 125 and name   HA) 3.98 2.29 2.28
assign (resid 125 and name HG2*) (resid 127 and name  HB1) 4.32 2.56 2.56
assign (resid 125 and name   HB) (resid 125 and name   HA) 3.66 1.96 1.96
assign (resid 126 and name  HA2) (resid 126 and name  HA1) 3.45 1.75 1.75
assign (resid 127 and name   HA) (resid 127 and name  HG1) 4.54 2.46 2.46
assign (resid 127 and name   HA) (resid 127 and name  HB1) 3.95 2.12 2.12
assign (resid 127 and name   HA) (resid 127 and name  HB2) 4.21 2.27 2.28
assign (resid 127 and name  HG2) (resid 127 and name   HA) 4.57 2.47 2.46
assign (resid 127 and name  HG2) (resid 127 and name  HB2) 3.98 2.14 2.15
assign (resid 127 and name  HG1) (resid 127 and name  HB2) 3.92 2.12 2.12
assign (resid 127 and name  HG2) (resid 127 and name  HB1) 3.71 2.00 2.00
assign (resid 127 and name  HG1) (resid 127 and name  HB1) 3.47 1.77 1.77
assign (resid 128 and name   HA) (resid 128 and name  HB1) 4.01 2.16 2.16
assign (resid 128 and name   HA) (resid 128 and name  HB2) 4.08 2.20 2.20
assign (resid 128 and name  HB2) (resid 128 and name  HB1) 3.15 1.45 1.44
assign (resid 129 and name HG1*) (resid 123 and name  HG2) 5.08 2.97 2.97
assign (resid 129 and name HG1*) (resid 129 and name   HA) 4.24 2.52 2.52
assign (resid 129 and name HG1*) (resid 129 and name   HB) 4.05 2.35 2.35
assign (resid 129 and name HG1*) (resid 122 and name  HB1) 4.60 2.71 2.71
assign (resid 129 and name HG2*) (resid 129 and name   HA) 4.08 2.38 2.38
assign (resid 129 and name HG2*) (resid 133 and name  HB1) 5.13 3.00 3.00
assign (resid 129 and name HG2*) (resid 134 and name   HA) 5.17 3.02 3.02
assign (resid 129 and name HG2*) (resid 129 and name   HB) 4.07 2.37 2.37
assign (resid 129 and name HG2*) (resid 122 and name  HB2) 4.42 2.62 2.62
assign (resid 129 and name   HB) (resid 129 and name   HA) 4.31 2.32 2.33
assign (resid 130 and name HG2*) (resid 130 and name   HA) 3.90 2.20 2.20
assign (resid 130 and name HG2*) (resid 130 and name   HB) 3.97 2.27 2.27
assign (resid 130 and name HD1*) (resid 130 and name   HB) 4.83 2.83 2.83
assign (resid 130 and name   HB) (resid 130 and name   HA) 3.84 2.07 2.08
assign (resid 131 and name   HA) (resid 131 and name  HG2) 4.39 2.37 2.37
assign (resid 131 and name   HA) (resid 131 and name  HB1) 3.77 2.03 2.03
assign (resid 131 and name   HA) (resid 134 and name HD1*) 4.49 2.65 2.64
assign (resid 131 and name  HB1) (resid 131 and name  HG1) 3.06 1.36 1.35
assign (resid 131 and name  HB2) (resid 131 and name  HG2) 3.69 1.99 1.98
assign (resid 131 and name  HB1) (resid 131 and name  HG2) 3.84 2.07 2.07
assign (resid 132 and name  HG2) (resid 132 and name   HA) 3.79 2.04 2.04
assign (resid 132 and name  HG1) (resid 132 and name   HA) 4.44 2.39 2.39
assign (resid 133 and name   HA) (resid 133 and name  HB1) 4.39 2.37 2.37
assign (resid 133 and name   HA) (resid 136 and name  HB1) 4.28 2.31 2.31
assign (resid 133 and name   HA) (resid 133 and name  HB2) 3.95 2.13 2.13
assign (resid 133 and name   HA) (resid 136 and name  HB2) 3.96 2.14 2.14
assign (resid 133 and name  HB1) (resid 133 and name  HB2) 3.87 2.17 2.17
assign (resid 133 and name  HB2) (resid 129 and name HG2*) 5.04 2.95 2.95
assign (resid 134 and name HG2*) (resid 134 and name   HA) 4.33 2.57 2.56
assign (resid 134 and name HG2*) (resid 134 and name   HB) 4.02 2.32 2.33
assign (resid 134 and name HD1*) (resid 123 and name  HD1) 5.24 3.06 3.06
assign (resid 134 and name HD1*) (resid 134 and name   HB) 5.10 2.98 2.98
assign (resid 134 and name HD1*) (resid 122 and name  HB2) 4.70 2.77 2.76
assign (resid 134 and name   HB) (resid 131 and name   HA) 4.42 2.62 2.62
assign (resid 134 and name   HA) (resid 134 and name   HB) 4.65 2.51 2.51
assign (resid 134 and name   HA) (resid 137 and name  HB2) 4.61 2.49 2.48
assign (resid 135 and name   HA) (resid 115 and name HD1*) 4.25 2.52 2.53
assign (resid 135 and name   HA) (resid 138 and name  HB2) 4.65 2.51 2.51
assign (resid 135 and name  HB2) (resid 135 and name   HA) 3.57 1.87 1.87
assign (resid 135 and name  HB1) (resid 132 and name   HA) 3.67 1.97 1.96
assign (resid 136 and name  HB1) (resid 136 and name   HA) 4.29 2.32 2.31
assign (resid 136 and name  HB2) (resid 136 and name   HA) 4.08 2.20 2.20
assign (resid 136 and name  HB1) (resid 136 and name  HB2) 3.22 1.52 1.52
assign (resid 137 and name   HG) (resid 137 and name   HA) 4.15 2.24 2.24
assign (resid 137 and name   HG) (resid 137 and name  HB1) 3.54 1.84 1.84
assign (resid 137 and name HD1*) (resid 137 and name  HB1) 4.46 2.64 2.64
assign (resid 137 and name HD1*) (resid 137 and name   HA) 4.86 2.85 2.85
assign (resid 137 and name HD1*) (resid 137 and name   HG) 4.49 2.65 2.64
assign (resid 137 and name HD2*) (resid 134 and name   HA) 4.96 2.91 2.91
assign (resid 137 and name HD2*) (resid 137 and name  HB1) 4.10 2.40 2.41
assign (resid 137 and name HD2*) (resid 137 and name  HB2) 4.38 2.59 2.60
assign (resid 137 and name HD2*) (resid 137 and name   HG) 4.27 2.53 2.54
assign (resid 137 and name  HB2) (resid 137 and name   HA) 4.33 2.33 2.33
assign (resid 137 and name  HB1) (resid 137 and name   HA) 4.45 2.40 2.40
assign (resid 138 and name  HE*) (resid 148 and name   HA) 3.97 2.27 2.27
assign (resid 138 and name  HB1) (resid 138 and name  HE*) 4.45 2.62 2.62
assign (resid 138 and name  HB2) (resid 138 and name  HG1)  4.54  2.46  2.46
assign (resid 138 and name  HB1) (resid 138 and name  HG1)  4.49  2.42  2.42
assign (resid 138 and name  HB1) (resid 138 and name  HG2)  4.37  2.36  2.36
assign (resid 138 and name  HG1) (resid 138 and name  HG2)  3.29  1.59  1.59
assign (resid 138 and name   HA) (resid 149 and name HD1*)  4.29  2.54  2.54
assign (resid 138 and name   HA) (resid 137 and name  HB2)  4.47  2.41  2.42
assign (resid 139 and name  HG2) (resid 139 and name   HA)  4.37  2.36  2.35
assign (resid 139 and name  HG1) (resid 139 and name   HA)  4.58  2.48  2.47
assign (resid 139 and name  HG2) (resid 139 and name  HG1)  3.26  1.56  1.56
assign (resid 139 and name   HA) (resid 138 and name  HG2)  3.97  2.14  2.14
assign (resid 140 and name   HA) (resid 140 and name  HB2)  4.04  2.18  2.18
assign (resid 141 and name  HB1) (resid 141 and name   HA)  4.08  2.21  2.21
assign (resid 141 and name  HB2) (resid 141 and name   HA)  4.62  2.50  2.50
assign (resid 141 and name  HB2) (resid 157 and name  HG2)  4.39  2.37  2.37
assign (resid 141 and name  HB2) (resid 149 and name HD1*)  5.04  2.95  2.95
assign (resid 142 and name   HA) (resid 142 and name  HB1)  4.44  2.40  2.39
assign (resid 142 and name   HA) (resid 142 and name  HB2)  4.17  2.25  2.25
assign (resid 142 and name  HB1) (resid 142 and name  HB2)  3.77  2.04  2.03
assign (resid 143 and name   HA) (resid 143 and name  HD1)  4.00  2.16  2.16
assign (resid 143 and name   HA) (resid 143 and name  HD2)  3.86  2.08  2.08
assign (resid 143 and name   HA) (resid 143 and name  HG2)  4.00  2.16  2.16
assign (resid 143 and name   HA) (resid 143 and name  HG1)  4.37  2.36  2.36
assign (resid 143 and name  HG2) (resid 156 and name  HE1)  4.61  2.49  2.48
assign (resid 143 and name  HG1) (resid 143 and name  HD2)  3.56  1.86  1.86
assign (resid 143 and name  HG1) (resid 143 and name  HG2)  3.35  1.66  1.65
assign (resid 144 and name   HA) (resid 144 and name  HB1)  3.81  2.06  2.06
assign (resid 144 and name   HA) (resid 144 and name  HB2)  3.91  2.11  2.11
assign (resid 145 and name   HA) (resid 145 and name  HB1)  3.73  2.01  2.02
assign (resid 145 and name   HA) (resid 145 and name  HB2)  4.08  2.20  2.20
assign (resid 145 and name  HB1) (resid 145 and name  HB2)  3.39  1.69  1.69
assign (resid 146 and name   HA) (resid 146 and name  HB1)  3.89  2.10  2.10
assign (resid 146 and name   HA) (resid 146 and name  HB2)  3.90  2.10  2.10
assign (resid 146 and name  HB2) (resid 146 and name  HB1)  3.75  2.02  2.02
assign (resid 147 and name  HA2) (resid 147 and name  HA1)  3.60  1.91  1.90
assign (resid 148 and name   HA) (resid 148 and name  HB1)  4.39  2.37  2.37
assign (resid 148 and name   HA) (resid 148 and name  HB2)  4.46  2.40  2.41
assign (resid 148 and name  HD2) (resid 148 and name  HD1)  3.49  1.79  1.79
assign (resid 148 and name  HD1) (resid 148 and name  HB1)  4.58  2.47  2.47
assign (resid 148 and name  HB2) (resid 148 and name  HG1)  4.04  2.17  2.17
assign (resid 148 and name  HB2) (resid 148 and name  HG2)  4.34  2.34  2.34
assign (resid 148 and name  HB1) (resid 148 and name  HG1)  4.19  2.26  2.25
assign (resid 148 and name  HB1) (resid 148 and name  HB2)  3.58  1.88  1.88
assign (resid 149 and name HG2*) (resid 149 and name   HA)  4.50  2.66  2.66
assign (resid 149 and name HG2*) (resid 154 and name   HA)  4.81  2.82  2.83
assign (resid 149 and name HG2*) (resid 154 and name  HB1)  4.91  2.88  2.88
assign (resid 149 and name HG2*) (resid 149 and name   HB)  4.13  2.43  2.44
assign (resid 149 and name HG2*) (resid 149 and name HD1*)  4.51  2.81  2.82
assign (resid 149 and name HD1*) (resid 141 and name  HB1)  4.27  2.53  2.53
assign (resid 149 and name HD1*) (resid 149 and name   HA)  4.88  2.86  2.86
assign (resid 149 and name HD1*) (resid 149 and name   HB)  4.80  2.82  2.83
assign (resid 149 and name HD1*) (resid 157 and name  HE*)  4.57  2.87  2.87
assign (resid 149 and name   HA) (resid 148 and name  HB1)  4.51  2.43  2.43
assign (resid 149 and name   HA) (resid 149 and name   HB)  4.67  2.52  2.51
assign (resid 150 and name   HA) (resid 150 and name  HB1)  4.19  2.26  2.25
assign (resid 150 and name   HA) (resid 150 and name  HB2)  4.32  2.33  2.33
assign (resid 150 and name   HA) (resid 113 and name HG12)  4.69  2.53  2.53
assign (resid 150 and name  HB2) (resid 150 and name  HB1)  4.01  2.31  2.32
assign (resid 151 and name  HB2) (resid 151 and name  HB1)  3.74  2.02  2.02
assign (resid 152 and name  HB2) (resid 152 and name   HA)  4.12  2.22  2.22
assign (resid 152 and name  HB1) (resid 152 and name   HA)  3.96  2.13  2.13
assign (resid 152 and name  HB1) (resid 152 and name  HB2)  3.87  2.17  2.17
assign (resid 152 and name   HA) (resid 155 and name HD1*)  4.66  2.74  2.74
assign (resid 154 and name  HB2) (resid 149 and name HG2*) 5.19 3.03 3.03
assign (resid 154 and name  HB2) (resid 155 and name   HG) 4.58 2.48 2.47
assign (resid 154 and name  HB1) (resid 154 and name  HB2) 4.20 2.50 2.50
assign (resid 155 and name   HG) (resid 155 and name HD1*) 4.61 2.72 2.72
assign (resid 155 and name   HG) (resid 155 and name HD2*) 4.52 2.67 2.67
assign (resid 155 and name HD1*) (resid 155 and name   HA) 4.11 2.41 2.41
assign (resid 155 and name HD1*) (resid 155 and name  HB1) 5.03 2.94 2.94
assign (resid 155 and name  HB2) (resid 155 and name   HA) 4.47 2.41 2.42
assign (resid 155 and name  HB1) (resid 155 and name   HA) 4.58 2.48 2.47
assign (resid 155 and name  HB2) (resid 155 and name  HB1) 3.67 1.97 1.96
assign (resid 155 and name  HB1) (resid 155 and name   HG) 3.92 2.11 2.12
assign (resid 155 and name  HB1) (resid 155 and name HD2*) 4.96 2.91 2.91
assign (resid 155 and name  HB2) (resid 155 and name HD2*) 4.73 2.78 2.78
assign (resid 155 and name  HB2) (resid 155 and name HD1*) 4.86 2.85 2.85
assign (resid 155 and name   HA) (resid 155 and name HD2*) 4.21 2.50 2.50
assign (resid 155 and name   HA) (resid 155 and name   HG) 4.63 2.50 2.50
assign (resid 155 and name   HA) (resid 158 and name  HB2) 4.60 2.48 2.47
assign (resid 156 and name  HD2) (resid 156 and name   HA) 4.58 2.47 2.47
assign (resid 156 and name  HD1) (resid 156 and name   HA) 4.22 2.28 2.28
assign (resid 156 and name  HD1) (resid 153 and name   HA) 4.45 2.40 2.40
assign (resid 156 and name  HD1) (resid 156 and name  HE1) 4.28 2.31 2.30
assign (resid 156 and name  HD2) (resid 156 and name  HE1) 4.39 2.37 2.37
assign (resid 156 and name  HD2) (resid 156 and name  HD1) 3.53 1.83 1.83
assign (resid 156 and name  HD1) (resid 156 and name  HE2) 4.67 2.52 2.51
assign (resid 156 and name  HD2) (resid 156 and name  HE2) 4.68 2.52 2.52
assign (resid 156 and name  HE1) (resid 156 and name  HG2) 4.22 2.28 2.28
assign (resid 156 and name   HA) (resid 156 and name  HG1) 4.06 2.19 2.20
assign (resid 157 and name  HG1) (resid 157 and name  HE*) 5.07 2.96 2.96
assign (resid 157 and name  HG2) (resid 157 and name  HG1) 3.54 1.84 1.84
assign (resid 157 and name   HA) (resid 157 and name  HG2) 4.15 2.24 2.24
assign (resid 158 and name   HA) (resid 158 and name  HB1) 3.64 1.94 1.94
assign (resid 158 and name  HB2) (resid 158 and name   HA) 4.61 2.49 2.48
assign (resid 160 and name  HA2) (resid 160 and name  HA1) 3.47 1.77 1.77
assign (resid 161 and name HG1*) (resid 161 and name   HB) 4.39 2.60 2.60
assign (resid 161 and name HG1*) (resid 161 and name   HA) 4.33 2.57 2.56
assign (resid 161 and name HG1*) (resid 160 and name  HA1) 4.77 2.80 2.80
assign (resid 161 and name HG1*) (resid 160 and name  HA2) 4.83 2.83 2.83
assign (resid 161 and name   HA) (resid 160 and name  HA2) 4.11 2.22 2.21
assign (resid 161 and name   HA) (resid 161 and name   HB) 4.12 2.23 2.22
assign (resid 162 and name  HB2) (resid 162 and name  HB1) 3.29 1.59 1.59
assign (resid   4 and name   HN) (resid   2 and name   HA) 3.04 0.54 0.53
assign (resid   4 and name   HN) (resid   3 and name  HE*) 3.54 1.03 1.03
assign (resid   4 and name   HN) (resid   4 and name   HA) 2.83 0.58 0.58
assign (resid   4 and name   HN) (resid   4 and name HG2*) 3.54 1.04 1.04
assign (resid   5 and name   HN) (resid   6 and name   HN) 3.60 1.80 1.80
assign (resid   6 and name HE21) (resid   6 and name  HG1) 3.65 1.84 1.85
assign (resid   6 and name   HN) (resid   8 and name   HN) 4.02 1.63 1.63
assign (resid   6 and name HE22) (resid  68 and name  HB2) 3.77 1.53 1.53
assign (resid   7 and name   HN) (resid   4 and name   HB) 3.08 1.25 1.25
assign (resid   7 and name HE21) (resid   6 and name  HB2) 4.05 1.64 1.64
assign (resid   7 and name   HN) (resid   8 and name   HN) 3.92 1.58 1.58
assign (resid   7 and name   HN) (resid   8 and name  HB*) 4.27 2.03 2.03
assign (resid   8 and name   HN) (resid   7 and name   HA) 3.32 0.58 0.58
assign (resid   8 and name   HN) (resid   8 and name   HA) 2.92 0.50 0.50
assign (resid   9 and name   HN) (resid   6 and name   HA) 2.95 0.76 0.75
assign (resid   9 and name   HN) (resid   7 and name   HA) 2.78 0.92 0.92
assign (resid   9 and name   HN) (resid   8 and name  HB*) 3.04 0.94 0.94
assign (resid   9 and name   HN) (resid   8 and name   HA) 2.92 0.50 0.50
assign (resid   9 and name   HN) (resid   9 and name   HA) 2.92 0.50 0.50
assign (resid   9 and name   HN) (resid   9 and name  HG1) 2.63 0.46 0.46
assign (resid   9 and name   HN) (resid   9 and name  HB*) 2.65 0.88 0.88
assign (resid   9 and name   HN) (resid  11 and name   HN) 2.61 0.59 0.59
assign (resid 10 and name  HN) (resid  9 and name   HN) 2.95 1.15 1.15
assign (resid 10 and name  HN) (resid  9 and name  HB*) 3.47 1.02 1.02
assign (resid 10 and name  HN) (resid  9 and name   HA) 3.00 0.59 0.60
assign (resid 10 and name  HN) (resid 10 and name  HB*) 3.13 0.96 0.96
assign (resid 10 and name  HN) (resid 10 and name   HA) 2.92 0.50 0.50
assign (resid 10 and name  HN) (resid 83 and name HG1*) 4.43 1.19 1.18
assign (resid 11 and name  HN) (resid  7 and name   HA) 2.61 1.18 1.19
assign (resid 11 and name  HN) (resid 10 and name   HA) 2.77 1.26 1.27
assign (resid 11 and name  HN) (resid 10 and name  HB*) 3.08 1.68 1.67
assign (resid 11 and name  HN) (resid 11 and name  HD*) 3.99 2.09 2.09
assign (resid 11 and name  HN) (resid 11 and name  HB*) 2.90 1.59 1.59
assign (resid 11 and name  HN) (resid 11 and name  HG2) 2.79 1.27 1.27
assign (resid 11 and name  HN) (resid 12 and name   HN) 2.97 1.35 1.35
assign (resid 12 and name  HN) (resid  9 and name   HN) 3.95 0.69 0.69
assign (resid 12 and name  HN) (resid  9 and name   HA) 2.92 0.50 0.50
assign (resid 12 and name  HN) (resid 10 and name   HN) 3.74 0.65 0.65
assign (resid 12 and name  HN) (resid 11 and name   HA) 3.45 0.60 0.60
assign (resid 12 and name  HN) (resid 12 and name   HA) 2.92 0.50 0.50
assign (resid 13 and name  HN) (resid 10 and name   HA) 3.34 0.58 0.59
assign (resid 13 and name  HN) (resid 11 and name   HN) 3.08 0.61 0.62
assign (resid 13 and name  HN) (resid 11 and name  HB*) 3.96 1.10 1.11
assign (resid 13 and name  HN) (resid 12 and name   HA) 3.04 0.53 0.53
assign (resid 13 and name  HN) (resid 12 and name  HB*) 3.24 0.98 0.98
assign (resid 13 and name  HN) (resid 13 and name   HA) 2.92 0.50 0.50
assign (resid 13 and name  HN) (resid 13 and name  HB2) 2.55 0.44 0.45
assign (resid 13 and name  HN) (resid 13 and name  HB1) 2.59 0.45 0.45
assign (resid 13 and name  HN) (resid 14 and name   HN) 2.79 0.49 0.48
assign (resid 14 and name  HN) (resid 10 and name   HA) 3.75 1.52 1.52
assign (resid 14 and name  HN) (resid 13 and name  HB2) 3.42 1.62 1.62
assign (resid 14 and name  HN) (resid 13 and name  HB1) 3.50 1.70 1.70
assign (resid 14 and name  HN) (resid 14 and name  HB1) 3.40 1.38 1.38
assign (resid 14 and name  HN) (resid 14 and name  HB2) 3.09 1.25 1.25
assign (resid 14 and name  HN) (resid 14 and name HD1*) 4.00 1.92 1.92
assign (resid 14 and name  HN) (resid 83 and name HG2*) 4.37 2.07 2.06
assign (resid 15 and name  HN) (resid 14 and name   HN) 3.03 1.38 1.38
assign (resid 15 and name  HN) (resid 14 and name   HA) 2.77 1.26 1.27
assign (resid 15 and name  HN) (resid 14 and name  HB2) 3.10 1.41 1.41
assign (resid 15 and name  HN) (resid 18 and name   HN) 2.75 1.25 1.25
assign (resid 15 and name  HN) (resid 18 and name  HB1) 2.79 1.27 1.27
assign (resid 16 and name  HN) (resid 15 and name  HB2) 2.92 0.50 0.50
assign (resid 16 and name  HN) (resid 16 and name   HA) 2.92 0.50 0.50
assign (resid 16 and name  HN) (resid 16 and name  HB*) 3.13 0.96 0.96
assign (resid 16 and name  HN) (resid 17 and name   HN) 3.21 0.56 0.56
assign (resid 17 and name  HN) (resid 16 and name   HA) 3.50 0.61 0.61
assign (resid 17 and name  HN) (resid 16 and name  HG*) 4.04 1.54 1.54
assign (resid 17 and name  HN) (resid 16 and name  HB*) 3.32 0.99 0.99
assign (resid 17 and name  HN) (resid 17 and name   HA) 2.92 0.50 0.50
assign (resid 17 and name  HN) (resid 17 and name  HG2) 3.53 1.03 1.03
assign (resid 17 and name  HN) (resid 17 and name  HB1) 2.56 0.76 0.75
assign (resid 17 and name  HN) (resid 17 and name  HB2) 2.74 0.94 0.94
assign (resid 17 and name  HN) (resid 18 and name   HN) 3.06 0.81 0.81
assign (resid 18 and name  HN) (resid 15 and name  HB2) 2.89 1.17 1.17
assign (resid 18 and name  HN) (resid 17 and name  HB2) 3.03 1.23 1.23
assign (resid 18 and name  HN) (resid 17 and name  HB1) 3.25 1.31 1.32
assign (resid 18 and name  HN) (resid 18 and name  HG1) 3.24 1.31 1.31
assign (resid 18 and name  HN) (resid 18 and name  HG2) 3.26 1.32 1.33
assign (resid 18 and name  HN) (resid 18 and name  HB1) 2.97 1.20 1.21
assign (resid 18 and name  HN) (resid 18 and name  HB2) 3.09 1.25 1.25
assign (resid 18 and name  HN) (resid 19 and name   HN) 4.03 1.78 1.77
assign (resid 19 and name  HN) (resid 14 and name  HB2) 2.58 0.45 0.45
assign (resid 19 and name  HN) (resid 16 and name   HA) 2.90 0.50 0.50
assign (resid 19 and name  HN) (resid 17 and name   HN) 3.21 0.79 0.79
assign (resid 19 and name  HN) (resid 18 and name  HB1) 2.16 0.45 0.44
assign (resid 19 and name  HN) (resid 18 and name   HA) 2.66 0.84 0.84
assign (resid 19 and name  HN) (resid 19 and name   HA) 2.92 0.50 0.50
assign (resid 19 and name  HN) (resid 19 and name HD1*) 3.42 1.01 1.01
assign (resid 19 and name  HN) (resid 20 and name   HN) 2.48 0.43 0.43
assign (resid 20 and name  HN) (resid 17 and name   HA) 3.43 1.39 1.39
assign (resid 20 and name  HN) (resid 19 and name HG12) 4.02 1.63 1.63
assign (resid 20 and name  HN) (resid 20 and name  HB*) 3.37 1.66 1.66
assign (resid 21 and name  HN) (resid 18 and name   HA) 3.00 0.52 0.52
assign (resid 21 and name  HN) (resid 20 and name  HB*) 3.73 1.06 1.06
assign (resid 21 and name  HN) (resid 20 and name   HA) 2.99 0.62 0.61
assign (resid 21 and name  HN) (resid 21 and name   HA) 2.92 0.50 0.50
assign (resid 21 and name  HN) (resid 22 and name   HN) 3.12 0.62 0.62
assign (resid 22 and name  HN) (resid 19 and name   HA) 3.35 0.58 0.58
assign (resid 22 and name  HN) (resid 21 and name   HA) 3.20 0.56 0.55
assign (resid 22 and name  HN) (resid 22 and name   HA) 2.92 0.50 0.50
assign (resid 22 and name  HN) (resid 22 and name  HB1) 3.02 0.68 0.68
assign (resid 22 and name  HN) (resid 22 and name  HB2) 2.93 0.68 0.68
assign (resid 22 and name  HN) (resid 23 and name   HN) 2.85 0.50 0.50
assign (resid 23 and name  HN) (resid 19 and name HG2*) 3.86 1.09 1.09
assign (resid 23 and name  HN) (resid 20 and name   HA) 2.88 0.50 0.50
assign (resid 23 and name  HN) (resid 20 and name  HB*) 4.00 1.19 1.20
assign (resid 23 and name  HN) (resid 22 and name   HA) 3.34 0.58 0.59
assign (resid 23 and name  HN) (resid 22 and name  HB2) 3.13 0.55 0.55
assign (resid 23 and name  HN) (resid 23 and name   HA) 2.92 0.50 0.50
assign (resid 23 and name  HN) (resid 24 and name   HN) 2.91 0.51 0.50
assign (resid 23 and name  HN) (resid 75 and name   HZ) 3.08 0.54 0.54
assign (resid 24 and name  HN) (resid 21 and name   HA) 2.96 0.54 0.54
assign (resid 24 and name  HN) (resid 23 and name  HG*) 4.40 1.18 1.18
assign (resid 24 and name  HN) (resid 24 and name   HA) 2.92 0.50 0.50
assign (resid 24 and name  HN) (resid 25 and name   HN) 3.30 0.57 0.58
assign (resid 25 and name  HN) (resid 24 and name  HB*) 2.65 0.87 0.88
assign (resid 25 and name  HN) (resid 24 and name   HA) 2.80 0.69 0.70
assign (resid 25 and name  HN) (resid 25 and name   HA) 2.92 0.50 0.50
assign (resid 26 and name  HN) (resid 23 and name   HA) 3.65 0.64 0.64
assign (resid 26 and name  HN) (resid 24 and name   HN) 3.37 0.59 0.58
assign (resid 26 and name  HN) (resid 24 and name   HA) 4.42 0.77 0.78
assign (resid 26 and name  HN) (resid 25 and name   HA) 3.65 0.64 0.64
assign (resid 26 and name  HN) (resid 25 and name  HB*) 3.64 1.05 1.05
assign (resid 26 and name  HN) (resid 26 and name   HA) 2.92 0.50 0.50
assign (resid 26 and name  HN) (resid 26 and name  HB*) 3.56 1.31 1.31
assign (resid 26 and name  HN) (resid 27 and name   HN) 3.50 1.00 1.00
assign (resid 26 and name  HN) (resid 28 and name   HN) 4.23 0.74 0.74
assign (resid 27 and name  HN) (resid 25 and name   HN) 3.92 0.69 0.68
assign (resid 27 and name  HN) (resid 25 and name   HA) 3.93 0.69 0.69
assign (resid 27 and name  HN) (resid 26 and name   HA) 3.10 0.54 0.54
assign (resid 27 and name  HN) (resid 26 and name  HB*) 3.61 1.05 1.04
assign (resid 27 and name  HN) (resid 27 and name   HA) 2.92 0.50 0.50
assign (resid 27 and name  HN) (resid 27 and name  HB1) 2.84 1.04 1.04
assign (resid 27 and name  HN) (resid 28 and name   HN) 3.17 0.92 0.92
assign (resid 28 and name  HN) (resid 25 and name   HA) 3.03 0.62 0.62
assign (resid 28 and name  HN) (resid 27 and name   HA) 4.12 0.72 0.71
assign (resid 28 and name  HN) (resid 27 and name  HB2) 3.79 0.66 0.66
assign (resid 28 and name  HN) (resid 27 and name  HB1) 3.48 0.98 0.98
assign (resid 28 and name  HN) (resid 28 and name   HA) 2.92 0.50 0.50
assign (resid 28 and name  HN) (resid 28 and name  HB1) 2.94 0.69 0.69
assign (resid 28 and name  HN) (resid 28 and name  HB2) 2.94 0.69 0.69
assign (resid 28 and name  HN) (resid 29 and name   HN) 3.60 1.10 1.10
assign (resid 28 and name  HN) (resid 30 and name   HN) 4.06 1.06 1.06
assign (resid 29 and name  HN) (resid 26 and name   HA) 3.22 0.56 0.56
assign (resid 29 and name  HN) (resid 28 and name  HB2) 2.83 1.03 1.02
assign (resid 29 and name  HN) (resid 28 and name  HB1) 2.85 1.05 1.05
assign (resid 29 and name  HN) (resid 28 and name  HA) 3.01 0.69 0.69
assign (resid 29 and name  HN) (resid 28 and name  HG2) 2.95 0.70 0.70
assign (resid 29 and name  HN) (resid 29 and name  HA) 2.92 0.50 0.50
assign (resid 29 and name  HN) (resid 29 and name  HB1) 2.70 0.90 0.90
assign (resid 29 and name  HN) (resid 29 and name  HB2) 2.69 0.89 0.90
assign (resid 29 and name  HN) (resid 30 and name  HN) 2.81 0.50 0.50
assign (resid 29 and name  HN) (resid 37 and name HD1*) 4.10 1.13 1.13
assign (resid 29 and name  HN) (resid 45 and name HG1*) 3.87 1.09 1.09
assign (resid 29 and name  HN) (resid 45 and name HG2*) 4.14 1.13 1.14
assign (resid 30 and name  HN) (resid 27 and name  HA) 2.88 0.50 0.51
assign (resid 30 and name  HN) (resid 28 and name  HA) 3.34 0.65 0.66
assign (resid 30 and name  HN) (resid 28 and name  HB2) 3.88 1.23 1.22
assign (resid 30 and name  HN) (resid 29 and name  HA) 2.88 0.50 0.51
assign (resid 30 and name  HN) (resid 29 and name  HB1) 2.91 0.60 0.59
assign (resid 30 and name  HN) (resid 29 and name  HB2) 2.98 0.52 0.52
assign (resid 30 and name  HN) (resid 30 and name  HA) 2.92 0.50 0.50
assign (resid 30 and name  HN) (resid 30 and name  HB1) 2.48 0.44 0.44
assign (resid 30 and name  HN) (resid 30 and name  HB2) 2.56 0.45 0.45
assign (resid 30 and name  HN) (resid 31 and name  HN) 2.67 0.54 0.54
assign (resid 30 and name  HN) (resid 37 and name HD1*) 3.65 1.05 1.05
assign (resid 31 and name  HN) (resid 30 and name  HA) 2.85 0.50 0.50
assign (resid 31 and name  HN) (resid 30 and name  HB1) 3.45 0.65 0.65
assign (resid 31 and name  HN) (resid 30 and name  HB2) 3.93 0.69 0.69
assign (resid 31 and name  HN) (resid 31 and name  HA) 2.92 0.50 0.50
assign (resid 31 and name  HN) (resid 31 and name  HB*) 3.07 0.94 0.94
assign (resid 31 and name  HN) (resid 32 and name  HN) 3.19 0.94 0.94
assign (resid 31 and name  HN) (resid 33 and name  HN) 3.58 0.62 0.62
assign (resid 32 and name  HN) (resid 30 and name  HA) 3.14 1.43 1.43
assign (resid 32 and name  HN) (resid 31 and name  HA) 2.77 1.26 1.27
assign (resid 32 and name  HN) (resid 31 and name  HB*) 2.88 1.58 1.58
assign (resid 32 and name  HN) (resid 32 and name  HB1) 3.21 1.46 1.46
assign (resid 32 and name  HN) (resid 32 and name  HB2) 2.88 1.30 1.30
assign (resid 32 and name  HN) (resid 33 and name  HN) 2.49 1.13 1.13
assign (resid 32 and name  HN) (resid 33 and name  HA*) 3.80 2.00 2.00
assign (resid 33 and name  HN) (resid 33 and name  HA*) 3.29 1.63 1.63
assign (resid 33 and name  HN) (resid 34 and name  HN) 3.70 1.50 1.50
assign (resid 34 and name  HN) (resid 33 and name  HA*) 4.03 1.93 1.93
assign (resid 34 and name  HN) (resid 34 and name  HA1) 3.25 1.32 1.32
assign (resid 34 and name  HN) (resid 34 and name  HA2) 3.24 1.31 1.31
assign (resid 34 and name  HN) (resid 35 and name  HN) 3.36 1.36 1.36
assign (resid 35 and name  HN) (resid 35 and name  HA2) 3.53 1.43 1.43
assign (resid 35 and name  HN) (resid 35 and name  HA1) 3.88 1.57 1.58
assign (resid 35 and name  HN) (resid 36 and name  HN) 3.63 1.47 1.47
assign (resid 36 and name  HN) (resid 26 and name  HZ) 3.59 0.63 0.63
assign (resid 36 and name  HN) (resid 35 and name  HA1) 2.98 0.73 0.73
assign (resid 36 and name  HN) (resid 35 and name  HA2) 3.18 0.93 0.93
assign (resid 36 and name  HN) (resid 36 and name  HA) 2.92 0.50 0.50
assign (resid 36 and name  HN) (resid 36 and name  HB1) 3.50 0.61 0.62
assign (resid 37 and name  HN) (resid 26 and name  HZ) 2.98 0.52 0.52
assign (resid 37 and name  HN) (resid 36 and name  HA) 2.30 0.40 0.40
assign (resid 37 and name  HN) (resid 36 and name  HB1) 2.82 0.49 0.49
assign (resid 37 and name  HN) (resid 36 and name  HB2) 2.74 0.48 0.48
assign (resid 37 and name  HN) (resid 37 and name  HA) 2.92 0.50 0.50
assign (resid 37 and name  HN) (resid 37 and name  HB) 2.42 0.42 0.42
assign (resid 37 and name  HN) (resid 37 and name HG1*) 3.25 0.98 0.98
assign (resid 37 and name  HN) (resid 37 and name HG2*) 3.49 1.11 1.11
assign (resid 37 and name  HN) (resid 37 and name HD1*) 3.17 0.96 0.96
assign (resid 37 and name  HN) (resid 73 and name  HN) 2.92 0.57 0.58
assign (resid 37 and name  HN) (resid 73 and name  HB) 2.75 0.48 0.48
assign (resid 37 and name  HN) (resid 74 and name  HA) 2.95 0.52 0.51
assign (resid 38 and name  HN) (resid 37 and name HG2*) 3.80 2.00 2.00
assign (resid 38 and name  HN) (resid 37 and name  HA) 2.77 1.26 1.27
assign (resid  38 and name   HN) (resid  38 and name  HB2)  3.04  1.38  1.38
assign (resid  38 and name   HN) (resid  42 and name   HN)  3.78  1.72  1.71
assign (resid  39 and name   HN) (resid  38 and name   HA)  2.67  0.86  0.86
assign (resid  39 and name   HN) (resid  38 and name  HB2)  3.50  0.61  0.62
assign (resid  39 and name   HN) (resid  39 and name   HB)  2.75  0.95  0.95
assign (resid  39 and name   HN) (resid  39 and name   HA)  2.92  0.50  0.50
assign (resid  39 and name   HN) (resid  39 and name HG2*)  3.53  1.03  1.03
assign (resid  39 and name   HN) (resid  62 and name HD1*)  3.79  1.08  1.08
assign (resid  40 and name   HN) (resid  39 and name   HA)  3.65  0.64  0.64
assign (resid  40 and name   HN) (resid  40 and name   HA)  2.92  0.50  0.50
assign (resid  40 and name   HN) (resid  41 and name   HN)  2.83  0.50  0.50
assign (resid  41 and name   HN) (resid  40 and name   HA)  2.96  0.65  0.65
assign (resid  41 and name   HN) (resid  40 and name  HB2)  2.96  0.52  0.52
assign (resid  41 and name   HN) (resid  40 and name  HG1)  2.96  0.71  0.71
assign (resid  41 and name   HN) (resid  41 and name   HA)  2.92  0.50  0.50
assign (resid  41 and name   HN) (resid  42 and name   HN)  2.74  0.48  0.48
assign (resid  42 and name   HN) (resid  37 and name HG2*)  3.63  1.04  1.04
assign (resid  42 and name   HN) (resid  39 and name   HA)  3.52  0.61  0.61
assign (resid  42 and name   HN) (resid  41 and name   HA)  3.00  0.59  0.60
assign (resid  42 and name   HN) (resid  42 and name   HA)  2.92  0.50  0.50
assign (resid  42 and name   HN) (resid  42 and name  HB1)  2.70  0.90  0.90
assign (resid  42 and name   HN) (resid  42 and name   HG)  3.60  1.10  1.10
assign (resid  42 and name   HN) (resid  42 and name  HB2)  2.70  0.90  0.90
assign (resid  42 and name   HN) (resid  42 and name HD2*)  3.79  1.21  1.21
assign (resid  43 and name   HN) (resid  40 and name   HA)  2.77  1.26  1.27
assign (resid  43 and name   HN) (resid  42 and name   HA)  2.77  1.26  1.27
assign (resid  43 and name   HN) (resid  42 and name  HB1)  2.48  1.12  1.12
assign (resid  43 and name   HN) (resid  42 and name   HG)  3.13  1.42  1.42
assign (resid  43 and name   HN) (resid  42 and name  HB2)  2.80  1.27  1.27
assign (resid  43 and name   HN) (resid  43 and name  HA1)  2.23  1.01  1.01
assign (resid  43 and name   HN) (resid  43 and name  HA2)  2.41  1.10  1.09
assign (resid  43 and name   HN) (resid  44 and name   HN)  2.90  1.31  1.31
assign (resid  43 and name   HN) (resid  58 and name HD1*)  3.08  1.68  1.67
assign (resid  44 and name   HN) (resid  41 and name   HA)  3.32  0.58  0.58
assign (resid  44 and name   HN) (resid  42 and name  HB2)  4.22  0.87  0.88
assign (resid  44 and name   HN) (resid  43 and name  HA1)  2.92  0.50  0.50
assign (resid  44 and name   HN) (resid  43 and name  HA2)  3.58  0.62  0.62
assign (resid  44 and name   HN) (resid  44 and name   HB)  3.04  0.53  0.53
assign (resid  44 and name   HN) (resid  44 and name   HA)  2.92  0.50  0.50
assign (resid  44 and name   HN) (resid  44 and name HG2*)  4.16  1.14  1.14
assign (resid  44 and name   HN) (resid  45 and name   HN)  2.80  1.00  1.00
assign (resid  44 and name   HN) (resid  58 and name HD1*)  4.08  1.12  1.12
assign (resid  45 and name   HN) (resid  42 and name   HA)  3.33  0.58  0.58
assign (resid  45 and name   HN) (resid  44 and name   HB)  2.87  0.50  0.50
assign (resid  45 and name   HN) (resid  44 and name   HA)  3.50  0.61  0.62
assign (resid  45 and name   HN) (resid  44 and name HG2*)  3.77  1.07  1.07
assign (resid  45 and name   HN) (resid  45 and name   HA)  2.92  0.50  0.50
assign (resid  45 and name   HN) (resid  45 and name   HB)  2.54  0.45  0.44
assign (resid  45 and name   HN) (resid  45 and name HG2*)  3.72  1.06  1.06
assign (resid  45 and name   HN) (resid  46 and name   HN)  2.83  0.50  0.50
assign (resid  46 and name   HN) (resid  43 and name  HA1)  3.85  0.75  0.75
assign (resid  46 and name   HN) (resid  43 and name  HA2)  3.79  0.67  0.67
assign (resid  46 and name   HN) (resid  45 and name   HA)  3.73  0.65  0.65
assign (resid  46 and name   HN) (resid  45 and name   HB)  2.75  0.48  0.48
assign (resid  46 and name   HN) (resid  45 and name HG1*)  4.00  1.11  1.11
assign (resid  46 and name   HN) (resid  45 and name HG2*)  3.75  1.06  1.06
assign (resid  46 and name   HN) (resid  46 and name   HA)  2.92  0.50  0.50
assign (resid  46 and name   HN) (resid  46 and name  HG1)  2.58  0.88  0.88
assign (resid  46 and name   HN) (resid  46 and name  HG2)  2.65  0.85  0.85
assign (resid  46 and name   HN) (resid  46 and name  HB1)  2.94  0.69  0.69
assign (resid  46 and name   HN) (resid  46 and name  HB2)  2.94  0.69  0.69
assign (resid  46 and name   HN) (resid  47 and name   HN)  3.03  0.53  0.53
assign (resid 46 and name  HN) (resid 58 and name HD1*) 3.98 1.11 1.11
assign (resid 47 and name  HN) (resid 44 and name   HA) 3.16 0.64 0.64
assign (resid 47 and name  HN) (resid 46 and name  HG1) 2.90 1.10 1.10
assign (resid 47 and name  HN) (resid 46 and name   HA) 3.03 0.53 0.53
assign (resid 47 and name  HN) (resid 46 and name  HB2) 2.48 0.68 0.68
assign (resid 47 and name  HN) (resid 47 and name  HD*) 3.08 1.28 1.28
assign (resid 47 and name  HN) (resid 47 and name   HA) 2.92 0.50 0.50
assign (resid 47 and name  HN) (resid 47 and name  HB2) 2.71 0.80 0.79
assign (resid 47 and name  HN) (resid 47 and name  HG2) 3.00 1.20 1.20
assign (resid 47 and name  HN) (resid 47 and name  HB1) 2.71 0.78 0.79
assign (resid 47 and name  HN) (resid 48 and name   HN) 3.21 0.71 0.71
assign (resid 47 and name  HN) (resid 53 and name  HD2) 3.36 0.64 0.64
assign (resid 48 and name  HN) (resid 45 and name   HA) 2.78 0.52 0.52
assign (resid 48 and name  HN) (resid 46 and name   HA) 3.30 0.70 0.70
assign (resid 48 and name  HN) (resid 47 and name  HB2) 2.96 0.75 0.75
assign (resid 48 and name  HN) (resid 48 and name  HB2) 2.89 1.09 1.09
assign (resid 48 and name  HN) (resid 48 and name  HG2) 2.92 0.67 0.67
assign (resid 48 and name  HN) (resid 48 and name   HA) 2.92 0.50 0.50
assign (resid 48 and name  HN) (resid 48 and name  HB1) 2.94 1.14 1.15
assign (resid 49 and name  HN) (resid 48 and name   HN) 3.61 1.46 1.46
assign (resid 49 and name  HN) (resid 50 and name   HN) 3.38 1.37 1.37
assign (resid 50 and name  HN) (resid 47 and name  HG1) 3.40 1.60 1.60
assign (resid 50 and name  HN) (resid 48 and name   HN) 2.85 1.05 1.05
assign (resid 50 and name  HN) (resid 49 and name   HA) 2.92 1.12 1.12
assign (resid 50 and name  HN) (resid 49 and name  HB1) 2.92 1.12 1.12
assign (resid 50 and name  HN) (resid 49 and name  HB2) 2.93 1.13 1.13
assign (resid 50 and name  HN) (resid 49 and name HD2*) 3.42 1.62 1.62
assign (resid 50 and name  HN) (resid 50 and name  HA1) 2.60 0.80 0.79
assign (resid 50 and name  HN) (resid 50 and name  HA2) 2.52 0.72 0.73
assign (resid 50 and name  HN) (resid 51 and name   HN) 2.60 0.80 0.79
assign (resid 51 and name  HN) (resid 47 and name   HA) 2.56 0.76 0.75
assign (resid 51 and name  HN) (resid 49 and name  HB1) 3.03 0.53 0.53
assign (resid 51 and name  HN) (resid 49 and name HD2*) 3.84 1.08 1.08
assign (resid 51 and name  HN) (resid 50 and name  HA1) 2.90 0.50 0.50
assign (resid 51 and name  HN) (resid 50 and name  HA2) 2.91 0.51 0.51
assign (resid 51 and name  HN) (resid 51 and name   HA) 2.92 0.50 0.50
assign (resid 51 and name  HN) (resid 51 and name  HB1) 2.54 0.45 0.44
assign (resid 51 and name  HN) (resid 51 and name  HB2) 2.54 0.44 0.44
assign (resid 51 and name HE22) (resid 51 and name  HG*) 3.56 1.03 1.04
assign (resid 51 and name HE21) (resid 51 and name  HG*) 3.33 1.00 1.00
assign (resid 51 and name HE21) (resid 53 and name  HB2) 3.32 0.58 0.58
assign (resid 52 and name  HN) (resid 51 and name   HA) 2.59 0.59 0.59
assign (resid 52 and name  HN) (resid 51 and name  HB1) 2.92 0.67 0.68
assign (resid 52 and name  HN) (resid 52 and name   HA) 2.92 0.50 0.50
assign (resid 52 and name HD22) (resid 52 and name  HB1) 2.91 0.59 0.59
assign (resid 52 and name HD21) (resid 52 and name  HB1) 2.57 0.45 0.45
assign (resid 52 and name HD21) (resid 52 and name  HB2) 2.67 0.47 0.47
assign (resid 54 and name  HN) (resid 53 and name   HA) 2.41 0.42 0.42
assign (resid 54 and name  HN) (resid 53 and name  HB1) 3.15 0.90 0.90
assign (resid 54 and name  HN) (resid 53 and name  HB2) 2.88 0.62 0.62
assign (resid 54 and name  HN) (resid 54 and name   HB) 3.29 0.58 0.57
assign (resid 54 and name  HN) (resid 54 and name   HA) 2.92 0.50 0.50
assign (resid 54 and name  HN) (resid 54 and name HG2*) 3.33 1.00 1.00
assign (resid 54 and name  HN) (resid 57 and name   HN) 3.90 0.68 0.68
assign (resid 54 and name  HN) (resid 58 and name   HG) 3.83 0.67 0.67
assign (resid 55 and name  HN) (resid 54 and name   HB) 3.06 0.54 0.54
assign (resid 55 and name  HN) (resid 54 and name   HA) 2.60 0.80 0.79
assign (resid 55 and name  HN) (resid 54 and name HG2*) 3.79 1.29 1.29
assign (resid 55 and name  HN) (resid 55 and name   HA) 2.92 0.50 0.50
assign (resid 55 and name  HN) (resid 55 and name  HB1) 2.70 0.90 0.90
assign (resid 55 and name  HN) (resid 55 and name  HB2) 2.54 0.74 0.75
assign (resid 55 and name  HN) (resid 56 and name   HN) 3.12 0.62 0.62
assign (resid 56 and name  HN) (resid 54 and name   HB) 3.76 0.65 0.66
assign (resid 56 and name  HN) (resid 54 and name HG2*) 4.34 1.34 1.34
assign (resid 56 and name  HN) (resid 55 and name   HA) 3.58 0.62 0.62
assign (resid 56 and name  HN) (resid 55 and name  HB1) 2.96 0.52 0.52
assign (resid 56 and name  HN) (resid 55 and name  HB2) 2.90 0.65 0.65
assign (resid 56 and name  HN) (resid 55 and name  HG*) 4.08 1.12 1.12
assign (resid 56 and name  HN) (resid 56 and name   HA) 2.92 0.50 0.50
assign (resid 56 and name  HN) (resid 56 and name  HG2) 3.02 1.02 1.02
assign (resid 56 and name  HN) (resid 56 and name  HB2) 3.07 0.63 0.63
assign (resid 56 and name  HN) (resid 56 and name  HG1) 3.36 1.36 1.36
assign (resid 56 and name  HN) (resid 57 and name   HN) 2.97 0.72 0.72
assign (resid 57 and name  HN) (resid 54 and name HG2*) 3.25 0.98 0.98
assign (resid 57 and name  HN) (resid 55 and name   HA) 4.13 0.72 0.72
assign (resid 57 and name  HN) (resid 55 and name  HB2) 3.85 0.94 0.94
assign (resid 57 and name  HN) (resid 56 and name   HA) 2.98 0.52 0.52
assign (resid 57 and name  HN) (resid 56 and name  HG2) 2.56 0.76 0.77
assign (resid 57 and name  HN) (resid 56 and name  HB2) 3.29 0.72 0.71
assign (resid 57 and name  HN) (resid 57 and name   HA) 2.92 0.50 0.50
assign (resid 57 and name  HN) (resid 58 and name   HN) 2.92 0.67 0.67
assign (resid 57 and name  HN) (resid 58 and name  HB*) 3.92 1.10 1.10
assign (resid 58 and name  HN) (resid 55 and name   HA) 3.19 1.45 1.44
assign (resid 58 and name  HN) (resid 56 and name   HN) 3.78 1.72 1.72
assign (resid 58 and name  HN) (resid 57 and name   HA) 2.77 1.26 1.27
assign (resid 58 and name  HN) (resid 58 and name   HG) 2.61 1.19 1.19
assign (resid 58 and name  HN) (resid 58 and name HD2*) 3.35 1.80 1.80
assign (resid 58 and name  HN) (resid 58 and name  HB*) 3.21 1.73 1.73
assign (resid 58 and name  HN) (resid 59 and name   HN) 2.73 1.24 1.24
assign (resid 58 and name  HN) (resid 60 and name   HN) 3.37 1.53 1.53
assign (resid 59 and name  HN) (resid 39 and name HG2*) 3.77 1.07 1.07
assign (resid 59 and name  HN) (resid 55 and name   HA) 4.11 0.72 0.71
assign (resid 59 and name  HN) (resid 56 and name   HA) 3.20 0.56 0.55
assign (resid 59 and name  HN) (resid 58 and name   HA) 3.58 0.62 0.62
assign (resid 59 and name  HN) (resid 58 and name   HG) 4.03 0.88 0.88
assign (resid 59 and name  HN) (resid 58 and name HD2*) 4.28 1.22 1.22
assign (resid 59 and name  HN) (resid 59 and name   HA) 2.92 0.50 0.50
assign (resid 59 and name  HN) (resid 59 and name  HB1) 2.60 0.80 0.80
assign (resid 59 and name  HN) (resid 59 and name  HB2) 2.69 0.89 0.90
assign (resid 59 and name  HN) (resid 60 and name   HN) 2.96 0.52 0.52
assign (resid 59 and name  HN) (resid 61 and name   HB) 4.04 0.71 0.71
assign (resid 60 and name  HN) (resid 57 and name   HA) 3.34 0.58 0.59
assign (resid 60 and name  HN) (resid 58 and name   HA) 3.46 0.65 0.64
assign (resid 60 and name  HN) (resid 59 and name  HB1) 3.12 0.55 0.54
assign (resid 60 and name  HN) (resid 59 and name  HB2) 3.34 0.58 0.59
assign (resid 60 and name  HN) (resid 59 and name   HA) 3.55 0.62 0.62
assign (resid 60 and name  HN) (resid 60 and name   HA) 2.92 0.50 0.50
assign (resid 60 and name  HN) (resid 60 and name  HB*) 2.97 0.93 0.93
assign (resid 60 and name  HN) (resid 61 and name   HN) 2.88 0.63 0.63
assign (resid 60 and name  HN) (resid 61 and name   HB) 3.54 0.86 0.86
assign (resid 61 and name  HN) (resid 58 and name   HA) 3.08 0.54 0.54
assign (resid 61 and name  HN) (resid 60 and name  HB*) 3.34 0.99 1.00
assign (resid 61 and name  HN) (resid 61 and name   HA) 2.92 0.50 0.50
assign (resid 61 and name  HN) (resid 61 and name   HB) 2.50 0.70 0.69
assign (resid 61 and name  HN) (resid 61 and name HG11) 2.80 0.49 0.49
assign (resid 61 and name  HN) (resid 61 and name HG2*) 3.36 1.00 1.00
assign (resid 61 and name  HN) (resid 62 and name   HN) 2.63 0.46 0.46
assign (resid 62 and name  HN) (resid 59 and name   HA) 3.13 0.55 0.55
assign (resid 62 and name  HN) (resid 60 and name   HA) 3.35 0.65 0.65
assign (resid 62 and name  HN) (resid 61 and name HG2*) 3.54 1.04 1.04
assign (resid 62 and name  HN) (resid 61 and name   HA) 3.22 0.56 0.56
assign (resid 62 and name  HN) (resid 62 and name   HA) 2.92 0.50 0.50
assign (resid 62 and name  HN) (resid 62 and name HG11) 2.39 0.59 0.59
assign (resid 62 and name  HN) (resid 62 and name HG12) 2.61 0.81 0.81
assign (resid  62 and name   HN) (resid  63 and name   HN) 2.62 0.82 0.81
assign (resid  62 and name   HN) (resid  64 and name   HN) 3.52 0.61 0.61
assign (resid  63 and name   HN) (resid  60 and name   HA) 3.57 0.63 0.62
assign (resid  63 and name   HN) (resid  62 and name   HA) 3.73 0.65 0.65
assign (resid  63 and name   HN) (resid  62 and name   HB) 2.88 0.50 0.51
assign (resid  63 and name   HN) (resid  62 and name HG2*) 3.77 1.07 1.07
assign (resid  63 and name   HN) (resid  63 and name   HA) 2.92 0.50 0.50
assign (resid  63 and name   HN) (resid  63 and name  HB1) 2.56 0.76 0.76
assign (resid  63 and name   HN) (resid  63 and name  HB2) 2.60 0.80 0.79
assign (resid  63 and name   HN) (resid  64 and name   HN) 3.11 0.54 0.54
assign (resid  64 and name   HN) (resid  61 and name   HA) 3.78 0.66 0.66
assign (resid  64 and name   HN) (resid  63 and name   HA) 3.61 0.63 0.63
assign (resid  64 and name   HN) (resid  63 and name  HB1) 2.71 0.47 0.48
assign (resid  64 and name   HN) (resid  63 and name  HB2) 2.75 0.48 0.49
assign (resid  64 and name   HN) (resid  63 and name  HG2) 4.14 0.72 0.73
assign (resid  64 and name   HN) (resid  64 and name   HA) 2.92 0.50 0.50
assign (resid  64 and name   HN) (resid  64 and name  HB1) 2.50 0.70 0.69
assign (resid  64 and name   HN) (resid  64 and name  HB2) 2.52 0.72 0.72
assign (resid  65 and name   HN) (resid  62 and name   HA) 3.21 0.56 0.56
assign (resid  65 and name   HN) (resid  64 and name   HA) 3.32 0.58 0.58
assign (resid  65 and name   HN) (resid  64 and name  HB1) 2.90 0.65 0.65
assign (resid  65 and name   HN) (resid  64 and name  HB2) 2.83 0.58 0.57
assign (resid  65 and name   HN) (resid  65 and name   HA) 2.92 0.50 0.50
assign (resid  65 and name   HN) (resid  65 and name   HB) 2.90 0.50 0.50
assign (resid  65 and name   HN) (resid  65 and name HG1*) 3.29 1.21 1.21
assign (resid  65 and name   HN) (resid  66 and name   HN) 2.62 0.46 0.46
assign (resid  67 and name   HN) (resid  66 and name   HN) 3.00 0.70 0.70
assign (resid  67 and name   HN) (resid  67 and name   HA) 2.92 0.50 0.50
assign (resid  67 and name   HN) (resid  67 and name  HB1) 2.62 0.82 0.81
assign (resid  67 and name   HN) (resid  67 and name  HB2) 2.46 0.66 0.65
assign (resid  67 and name   HN) (resid  68 and name   HN) 2.79 0.49 0.48
assign (resid  67 and name   HN) (resid  69 and name   HN) 3.41 0.60 0.59
assign (resid  68 and name   HN) (resid  67 and name   HA) 3.25 0.56 0.56
assign (resid  68 and name   HN) (resid  67 and name  HB1) 3.18 0.68 0.68
assign (resid  68 and name   HN) (resid  67 and name  HB2) 3.20 0.56 0.56
assign (resid  68 and name   HN) (resid  68 and name   HA) 2.92 0.50 0.50
assign (resid  68 and name   HN) (resid  68 and name  HB1) 3.23 0.73 0.73
assign (resid  68 and name   HN) (resid  69 and name   HN) 2.74 0.48 0.48
assign (resid  69 and name   HN) (resid  68 and name  HB2) 2.76 1.25 1.25
assign (resid  69 and name   HN) (resid  68 and name   HA) 2.77 1.26 1.27
assign (resid  69 and name   HN) (resid  69 and name  HA1) 2.17 0.99 0.98
assign (resid  69 and name   HN) (resid  69 and name  HA2) 2.01 0.91 0.91
assign (resid  69 and name   HN) (resid  70 and name   HN) 2.31 1.05 1.06
assign (resid  70 and name   HN) (resid  66 and name  HB*) 4.12 1.62 1.62
assign (resid  70 and name   HN) (resid  66 and name   HA) 3.65 1.15 1.15
assign (resid  70 and name   HN) (resid  68 and name   HN) 3.71 0.65 0.65
assign (resid  70 and name   HN) (resid  69 and name  HA1) 3.35 0.85 0.85
assign (resid  70 and name   HN) (resid  69 and name  HA2) 3.31 0.81 0.81
assign (resid  70 and name   HN) (resid  70 and name   HA) 2.92 0.50 0.50
assign (resid  70 and name   HN) (resid  70 and name  HB2) 2.79 0.99 0.98
assign (resid  70 and name   HN) (resid  70 and name  HB1) 2.83 0.58 0.58
assign (resid  70 and name   HN) (resid  71 and name   HN) 2.90 0.65 0.65
assign (resid  71 and name   HN) (resid  62 and name HG2*) 4.46 2.10 2.11
assign (resid  71 and name   HN) (resid  69 and name  HA2) 4.01 1.62 1.62
assign (resid  71 and name   HN) (resid  71 and name  HA1) 3.24 1.31 1.31
assign (resid  71 and name   HN) (resid  71 and name  HA2) 3.06 1.24 1.24
assign (resid  71 and name   HN) (resid  72 and name   HN) 3.42 1.39 1.38
assign (resid  72 and name   HN) (resid  62 and name HG2*) 3.73 1.11 1.11
assign (resid  72 and name   HN) (resid  70 and name  HB1) 3.23 0.56 0.56
assign (resid  72 and name   HN) (resid  71 and name  HA2) 2.95 0.70 0.69
assign (resid  72 and name   HN) (resid  71 and name  HA1) 2.92 0.67 0.67
assign (resid  72 and name   HN) (resid  72 and name   HA) 2.92 0.50 0.50
assign (resid 72 and name  HN) (resid 72 and name HG2*) 3.29 0.98 0.99
assign (resid 73 and name  HN) (resid 37 and name   HB) 2.88 0.50 0.50
assign (resid 73 and name  HN) (resid 38 and name   HA) 2.43 0.47 0.47
assign (resid 73 and name  HN) (resid 62 and name HD1*) 3.56 1.03 1.04
assign (resid 73 and name  HN) (resid 72 and name   HA) 2.34 0.41 0.41
assign (resid 73 and name  HN) (resid 72 and name   HB) 2.44 0.64 0.64
assign (resid 73 and name  HN) (resid 72 and name HG2*) 3.61 1.04 1.04
assign (resid 73 and name  HN) (resid 73 and name   HA) 2.92 0.50 0.50
assign (resid 73 and name  HN) (resid 73 and name   HB) 2.54 0.44 0.45
assign (resid 73 and name  HN) (resid 73 and name HG11) 2.48 0.68 0.67
assign (resid 73 and name  HN) (resid 73 and name HG2*) 3.52 1.02 1.02
assign (resid 73 and name  HN) (resid 73 and name HG12) 2.73 0.48 0.48
assign (resid 74 and name  HN) (resid 37 and name   HN) 3.45 1.06 1.05
assign (resid 74 and name  HN) (resid 73 and name   HA) 2.04 0.36 0.35
assign (resid 74 and name  HN) (resid 73 and name   HB) 2.95 0.76 0.75
assign (resid 74 and name  HN) (resid 73 and name HG2*) 3.09 0.95 0.96
assign (resid 74 and name  HN) (resid 74 and name   HA) 2.92 0.50 0.50
assign (resid 74 and name  HN) (resid 74 and name  HB1) 3.05 0.65 0.65
assign (resid 74 and name  HN) (resid 74 and name  HB2) 3.01 0.69 0.69
assign (resid 75 and name  HN) (resid 26 and name   HZ) 3.21 0.56 0.56
assign (resid 75 and name  HN) (resid 36 and name   HA) 3.49 0.61 0.61
assign (resid 75 and name  HN) (resid 74 and name   HA) 2.69 0.47 0.47
assign (resid 75 and name  HN) (resid 74 and name  HB2) 2.59 0.79 0.79
assign (resid 75 and name  HN) (resid 74 and name  HB1) 2.44 0.64 0.63
assign (resid 75 and name  HN) (resid 75 and name  HB2) 2.58 0.45 0.45
assign (resid 75 and name  HN) (resid 75 and name  HB1) 2.66 0.47 0.46
assign (resid 75 and name  HN) (resid 75 and name   HA) 2.92 0.50 0.50
assign (resid 75 and name  HN) (resid 76 and name   HN) 3.06 0.54 0.54
assign (resid 76 and name  HN) (resid 74 and name  HB1) 3.56 1.62 1.62
assign (resid 76 and name  HN) (resid 75 and name   HA) 2.77 1.26 1.27
assign (resid 76 and name  HN) (resid 75 and name  HB1) 3.27 1.49 1.49
assign (resid 76 and name  HN) (resid 75 and name  HB2) 2.60 1.18 1.18
assign (resid 76 and name  HN) (resid 77 and name   HN) 2.76 1.25 1.25
assign (resid 77 and name  HN) (resid 74 and name   HN) 3.58 0.62 0.62
assign (resid 77 and name  HN) (resid 74 and name  HB1) 2.75 0.48 0.48
assign (resid 77 and name  HN) (resid 75 and name   HA) 3.17 0.72 0.73
assign (resid 77 and name  HN) (resid 76 and name   HA) 3.60 0.63 0.63
assign (resid 77 and name  HN) (resid 77 and name   HA) 2.92 0.50 0.50
assign (resid 77 and name  HN) (resid 78 and name   HN) 2.63 0.46 0.46
assign (resid 77 and name  HN) (resid 78 and name  HB2) 3.75 1.00 1.00
assign (resid 77 and name  HN) (resid 78 and name  HB1) 3.24 0.97 0.96
assign (resid 78 and name  HN) (resid 73 and name HG2*) 3.58 1.75 1.75
assign (resid 78 and name  HN) (resid 75 and name   HA) 3.50 1.42 1.42
assign (resid 78 and name  HN) (resid 76 and name   HN) 4.00 1.62 1.62
assign (resid 78 and name  HN) (resid 78 and name  HB1) 2.99 1.21 1.21
assign (resid 78 and name  HN) (resid 78 and name  HB2) 2.89 1.17 1.17
assign (resid 78 and name  HN) (resid 79 and name   HG) 3.89 1.57 1.58
assign (resid 78 and name  HN) (resid 80 and name   HN) 4.16 1.69 1.68
assign (resid 79 and name  HN) (resid 76 and name   HA) 2.30 0.59 0.60
assign (resid 79 and name  HN) (resid 77 and name   HN) 2.77 0.93 0.93
assign (resid 79 and name  HN) (resid 78 and name   HA) 2.67 0.83 0.83
assign (resid 79 and name  HN) (resid 79 and name   HA) 2.92 0.50 0.50
assign (resid 79 and name  HN) (resid 80 and name   HN) 2.81 0.49 0.49
assign (resid 80 and name  HN) (resid 77 and name   HN) 3.62 1.08 1.08
assign (resid 80 and name  HN) (resid 79 and name   HA) 3.78 0.66 0.66
assign (resid 80 and name  HN) (resid 79 and name  HB1) 3.35 0.58 0.58
assign (resid 80 and name  HN) (resid 80 and name   HA) 2.92 0.50 0.50
assign (resid 80 and name  HN) (resid 80 and name   HB) 2.50 0.70 0.70
assign (resid 80 and name  HN) (resid 80 and name HG1*) 3.06 0.94 0.94
assign (resid 80 and name  HN) (resid 80 and name HG2*) 3.66 1.06 1.05
assign (resid 80 and name  HN) (resid 83 and name   HB) 3.79 0.72 0.71
assign (resid 81 and name  HN) (resid 78 and name   HA) 3.46 1.40 1.39
assign (resid 81 and name  HN) (resid 79 and name  HB2) 3.48 1.41 1.41
assign (resid 81 and name  HN) (resid 80 and name   HN) 3.00 1.21 1.21
assign (resid 81 and name  HN) (resid 80 and name   HB) 2.80 1.13 1.14
assign (resid 81 and name  HN) (resid 81 and name  HB1) 2.97 1.20 1.21
assign (resid 81 and name  HN) (resid 81 and name  HG2) 2.84 1.15 1.15
assign (resid 81 and name  HN) (resid 83 and name   HB) 3.12 1.39 1.38
assign (resid 82 and name  HN) (resid 79 and name   HA) 3.15 0.55 0.55
assign (resid 82 and name  HN) (resid 79 and name  HB2) 4.02 1.28 1.28
assign (resid 82 and name  HN) (resid 81 and name   HN) 2.73 0.48 0.48
assign (resid 82 and name  HN) (resid 81 and name   HA) 3.38 0.59 0.59
assign (resid 82 and name  HN) (resid 81 and name  HB1) 2.86 0.50 0.50
assign (resid 82 and name  HN) (resid 81 and name  HB2) 3.31 0.69 0.69
assign (resid 82 and name  HN) (resid 82 and name   HA) 2.92 0.50 0.50
assign (resid 82 and name  HN) (resid 82 and name  HB1) 2.54 0.44 0.44
assign (resid 82 and name  HN) (resid 82 and name  HB2) 2.74 0.64 0.63
assign (resid 82 and name  HN) (resid 83 and name   HN) 2.83 0.58 0.58
assign (resid 83 and name  HN) (resid 80 and name   HA) 3.26 0.57 0.57
assign (resid 83 and name  HN) (resid 82 and name  HB1) 3.12 0.54 0.54
assign (resid 83 and name  HN) (resid 82 and name  HB2) 3.69 0.65 0.65
assign (resid 83 and name  HN) (resid 83 and name   HA) 2.92 0.50 0.50
assign (resid 83 and name  HN) (resid 83 and name   HB) 2.42 0.61 0.61
assign (resid 83 and name  HN) (resid 83 and name HG1*) 3.17 1.03 1.03
assign (resid 83 and name  HN) (resid 83 and name HG2*) 3.65 1.05 1.05
assign (resid 83 and name  HN) (resid 84 and name   HN) 3.00 0.71 0.71
assign (resid 84 and name  HN) (resid 81 and name   HA) 2.92 0.50 0.50
assign (resid 84 and name  HN) (resid 83 and name   HA) 3.08 0.54 0.54
assign (resid 84 and name  HN) (resid 83 and name HG1*) 3.86 1.09 1.08
assign (resid 84 and name  HN) (resid 83 and name HG2*) 3.93 1.10 1.10
assign (resid 84 and name  HN) (resid 84 and name   HA) 2.92 0.50 0.50
assign (resid 84 and name  HN) (resid 84 and name  HB*) 3.10 0.95 0.96
assign (resid 84 and name  HN) (resid 85 and name   HN) 2.94 1.14 1.15
assign (resid 84 and name  HN) (resid 86 and name   HN) 3.35 0.85 0.85
assign (resid 85 and name  HN) (resid 84 and name  HB*) 3.22 1.08 1.08
assign (resid 85 and name  HN) (resid 84 and name  HG1) 2.64 0.84 0.84
assign (resid 85 and name  HN) (resid 85 and name   HA) 2.92 0.50 0.50
assign (resid 85 and name  HN) (resid 85 and name  HB*) 2.97 0.93 0.93
assign (resid 85 and name HE22) (resid 85 and name  HG1) 2.97 0.52 0.52
assign (resid 85 and name HE21) (resid 85 and name  HG1) 2.67 0.46 0.46
assign (resid 85 and name  HN) (resid 87 and name   HN) 2.96 0.52 0.52
assign (resid 86 and name  HN) (resid 87 and name   HN) 3.62 1.47 1.46
assign (resid 86 and name  HN) (resid 87 and name  HG2) 4.09 1.66 1.66
assign (resid 87 and name  HN) (resid 86 and name   HA) 2.77 1.26 1.27
assign (resid 87 and name  HN) (resid 87 and name  HG1) 2.79 1.27 1.26
assign (resid 87 and name  HN) (resid 88 and name   HN) 2.78 0.98 0.98
assign (resid 88 and name  HN) (resid 87 and name  HG1) 3.13 1.33 1.33
assign (resid 88 and name  HN) (resid 87 and name   HA) 2.92 0.50 0.50
assign (resid 88 and name  HN) (resid 88 and name   HA) 2.92 0.50 0.50
assign (resid 88 and name  HN) (resid 88 and name  HB1) 2.97 0.52 0.52
assign (resid 88 and name  HN) (resid 89 and name   HN) 3.01 0.76 0.76
assign (resid 89 and name  HN) (resid 88 and name   HA) 2.75 0.75 0.75
assign (resid 89 and name  HN) (resid 88 and name  HB1) 2.41 0.49 0.49
assign (resid 89 and name  HN) (resid 89 and name   HA) 2.92 0.50 0.50
assign (resid 89 and name  HN) (resid 89 and name  HB1) 2.21 0.49 0.49
assign (resid 89 and name  HN) (resid 89 and name  HB2) 2.21 0.49 0.49
assign (resid 89 and name  HN) (resid 90 and name   HN) 2.70 0.47 0.47
assign (resid 90 and name  HN) (resid 89 and name  HB1) 3.08 0.83 0.82
assign (resid 90 and name  HN) (resid 89 and name  HB2) 3.12 0.88 0.88
assign (resid 90 and name  HN) (resid 90 and name   HA) 2.92 0.50 0.50
assign (resid 90 and name  HN) (resid 90 and name  HB*) 3.80 1.55 1.55
assign (resid 91 and name  HN) (resid 90 and name   HA) 2.83 0.58 0.58
assign (resid 91 and name  HN) (resid 90 and name  HB*) 3.40 1.01 1.00
assign (resid 91 and name  HN) (resid 91 and name   HA) 2.92 0.50 0.50
assign (resid  91 and name   HN) (resid  91 and name  HB1)  2.81  0.81  0.81
assign (resid  91 and name   HN) (resid  92 and name   HN)  3.12  0.88  0.88
assign (resid  91 and name   HN) (resid  93 and name   HN)  3.06  0.65  0.65
assign (resid  92 and name   HN) (resid  91 and name   HA)  2.77  1.26  1.27
assign (resid  92 and name   HN) (resid  92 and name  HA*)  2.88  1.58  1.58
assign (resid  92 and name   HN) (resid  93 and name   HN)  3.14  1.43  1.43
assign (resid  92 and name   HN) (resid  93 and name  HB1)  2.93  1.37  1.37
assign (resid  93 and name   HN) (resid  92 and name  HA*)  3.30  0.99  0.99
assign (resid  93 and name   HN) (resid  93 and name   HA)  2.92  0.50  0.50
assign (resid  93 and name   HN) (resid  93 and name  HB2)  2.94  0.52  0.52
assign (resid  93 and name   HN) (resid  93 and name  HG1)  3.12  0.54  0.54
assign (resid  93 and name   HN) (resid  94 and name   HN)  3.11  0.86  0.86
assign (resid  94 and name   HN) (resid  93 and name   HA)  2.77  1.26  1.27
assign (resid  94 and name   HN) (resid  93 and name  HB1)  3.17  1.44  1.44
assign (resid  94 and name   HN) (resid  93 and name  HB2)  3.27  1.49  1.48
assign (resid  94 and name   HN) (resid  94 and name  HB1)  3.13  1.42  1.42
assign (resid  94 and name   HN) (resid  94 and name  HB2)  2.98  1.35  1.35
assign (resid  94 and name   HN) (resid  95 and name   HN)  3.17  1.44  1.44
assign (resid  95 and name   HN) (resid  92 and name  HA*)  3.29  0.98  0.99
assign (resid  95 and name   HN) (resid  93 and name   HN)  3.69  0.65  0.65
assign (resid  95 and name   HN) (resid  94 and name  HB1)  3.39  0.89  0.89
assign (resid  95 and name   HN) (resid  94 and name   HA)  3.07  0.54  0.54
assign (resid  95 and name   HN) (resid  95 and name   HA)  2.92  0.50  0.50
assign (resid  95 and name   HN) (resid  95 and name  HB*)  3.29  0.99  0.99
assign (resid  95 and name   HN) (resid  96 and name   HN)  3.13  0.88  0.88
assign (resid  96 and name   HN) (resid  96 and name   HA)  2.92  0.50  0.50
assign (resid  96 and name   HN) (resid  97 and name   HN)  3.32  1.07  1.07
assign (resid  96 and name   HN) (resid  99 and name   HN)  4.09  0.71  0.71
assign (resid  97 and name   HN) (resid  97 and name   HA)  2.92  0.50  0.50
assign (resid  97 and name   HN) (resid  97 and name  HB1)  2.60  0.80  0.80
assign (resid  97 and name   HN) (resid  98 and name   HN)  2.90  0.50  0.50
assign (resid  98 and name   HN) (resid  95 and name   HA)  3.25  0.56  0.56
assign (resid  98 and name   HN) (resid  97 and name   HA)  3.42  0.60  0.60
assign (resid  98 and name   HN) (resid  98 and name   HA)  2.92  0.50  0.50
assign (resid  98 and name   HN) (resid  99 and name   HN)  2.83  0.50  0.50
assign (resid  99 and name   HN) (resid  96 and name   HA)  3.21  0.79  0.79
assign (resid  99 and name   HN) (resid  98 and name   HA)  4.14  0.72  0.73
assign (resid  99 and name   HN) (resid  98 and name  HB1)  3.55  0.62  0.62
assign (resid  99 and name   HN) (resid  99 and name   HA)  2.92  0.50  0.50
assign (resid  99 and name   HN) (resid  99 and name  HB*)  3.26  0.98  0.98
assign (resid  99 and name   HN) (resid 100 and name   HN)  3.12  0.87  0.87
assign (resid  99 and name   HN) (resid 151 and name   HZ)  4.06  0.71  0.71
assign (resid 100 and name   HN) (resid  97 and name   HN)  3.91  1.09  1.09
assign (resid 100 and name   HN) (resid  97 and name   HA)  3.38  0.59  0.60
assign (resid 100 and name HD21) (resid  97 and name   HA)  3.75  1.25  1.25
assign (resid 100 and name   HN) (resid  98 and name   HA)  4.04  0.71  0.71
assign (resid 100 and name   HN) (resid  99 and name   HA)  3.46  0.60  0.60
assign (resid 100 and name   HN) (resid  99 and name  HB*)  3.83  1.08  1.08
assign (resid 100 and name   HN) (resid 100 and name   HA)  2.92  0.50  0.50
assign (resid 100 and name   HN) (resid 100 and name  HB1)  2.76  0.96  0.96
assign (resid 100 and name   HN) (resid 100 and name  HB2)  2.94  1.14  1.14
assign (resid 100 and name HD21) (resid 100 and name  HB2)  3.40  1.59  1.60
assign (resid 100 and name HD21) (resid 100 and name  HB1)  3.17  1.37  1.38
assign (resid 100 and name HD22) (resid 100 and name  HB1)  3.92  0.69  0.68
assign (resid 100 and name   HN) (resid 102 and name   HN)  3.97  0.69  0.70
assign (resid 101 and name   HN) (resid  98 and name   HN)  3.71  1.50  1.50
assign (resid 101 and name   HN) (resid 154 and name   HZ)  4.00  1.62  1.62
assign (resid 102 and name   HN) (resid  99 and name   HN)  3.86  0.74  0.74
assign (resid 102 and name   HN) (resid  99 and name   HA)  3.19  0.56  0.56
assign (resid 102 and name   HN) (resid 101 and name   HA)  3.40  0.60  0.60
assign (resid 102 and name   HN) (resid 101 and name  HB2)  2.77  0.48  0.48
assign (resid 102 and name   HN) (resid 102 and name   HA)  2.92  0.50  0.50
assign (resid 102 and name  HN) (resid 102 and name  HB1) 2.77 0.48 0.48
assign (resid 102 and name  HN) (resid 102 and name  HB2) 2.77 0.48 0.48
assign (resid 102 and name  HN) (resid 103 and name   HN) 2.99 0.74 0.73
assign (resid 102 and name  HN) (resid 154 and name   HZ) 3.37 0.59 0.58
assign (resid 103 and name  HN) (resid  99 and name   HA) 3.82 0.67 0.67
assign (resid 103 and name  HN) (resid 100 and name   HN) 3.80 0.90 0.90
assign (resid 103 and name  HN) (resid 100 and name   HA) 3.37 0.59 0.59
assign (resid 103 and name  HN) (resid 102 and name  HB1) 2.93 0.68 0.68
assign (resid 103 and name  HN) (resid 102 and name  HB2) 3.24 0.99 0.99
assign (resid 103 and name  HN) (resid 103 and name   HA) 2.92 0.50 0.50
assign (resid 103 and name  HN) (resid 103 and name  HB*) 3.04 0.94 0.95
assign (resid 103 and name  HN) (resid 104 and name   HN) 3.00 0.75 0.75
assign (resid 104 and name  HN) (resid 103 and name   HA) 3.33 0.58 0.58
assign (resid 104 and name  HN) (resid 103 and name  HB*) 3.65 1.05 1.05
assign (resid 104 and name  HN) (resid 104 and name   HA) 2.92 0.50 0.50
assign (resid 104 and name  HN) (resid 104 and name   HB) 2.48 0.68 0.68
assign (resid 104 and name  HN) (resid 104 and name HG11) 3.27 1.02 1.02
assign (resid 104 and name  HN) (resid 104 and name HG12) 3.04 0.79 0.78
assign (resid 104 and name  HN) (resid 104 and name HD1*) 4.21 1.15 1.15
assign (resid 104 and name  HN) (resid 104 and name HG2*) 3.82 1.08 1.08
assign (resid 105 and name  HN) (resid 102 and name   HA) 3.48 0.61 0.61
assign (resid 105 and name  HN) (resid 104 and name   HA) 3.62 0.64 0.63
assign (resid 105 and name  HN) (resid 104 and name   HB) 2.90 0.65 0.65
assign (resid 105 and name  HN) (resid 104 and name HG11) 3.82 0.67 0.67
assign (resid 105 and name  HN) (resid 104 and name HG2*) 3.91 1.10 1.09
assign (resid 105 and name  HN) (resid 105 and name   HA) 2.92 0.50 0.50
assign (resid 105 and name  HN) (resid 106 and name   HN) 2.58 0.78 0.78
assign (resid 105 and name  HN) (resid 113 and name HG11) 3.77 0.66 0.66
assign (resid 106 and name  HN) (resid 103 and name   HA) 2.67 0.52 0.53
assign (resid 106 and name  HN) (resid 105 and name   HA) 3.00 0.59 0.60
assign (resid 106 and name  HN) (resid 106 and name   HA) 2.92 0.50 0.50
assign (resid 106 and name  HN) (resid 106 and name  HB2) 3.00 0.75 0.75
assign (resid 106 and name  HN) (resid 113 and name HG11) 3.31 0.58 0.58
assign (resid 106 and name  HN) (resid 113 and name HD1*) 3.58 1.04 1.04
assign (resid 107 and name  HN) (resid 106 and name   HN) 3.69 0.64 0.64
assign (resid 107 and name  HN) (resid 106 and name   HA) 2.48 0.68 0.68
assign (resid 107 and name  HN) (resid 107 and name   HA) 2.92 0.50 0.50
assign (resid 107 and name  HN) (resid 107 and name  HB*) 3.22 0.98 0.98
assign (resid 107 and name  HN) (resid 107 and name  HD*) 3.91 1.10 1.09
assign (resid 107 and name  HN) (resid 107 and name  HG1) 2.79 0.99 1.00
assign (resid 107 and name  HN) (resid 107 and name  HG2) 3.02 0.77 0.77
assign (resid 107 and name  HN) (resid 108 and name   HN) 3.15 0.90 0.90
assign (resid 107 and name  HN) (resid 109 and name   HN) 3.80 0.66 0.67
assign (resid 107 and name  HN) (resid 117 and name  HG1) 3.40 0.90 0.90
assign (resid 108 and name  HN) (resid 107 and name   HA) 3.42 0.60 0.60
assign (resid 108 and name  HN) (resid 107 and name  HB*) 3.41 1.01 1.01
assign (resid 108 and name  HN) (resid 107 and name  HG1) 3.77 0.65 0.65
assign (resid 108 and name HD21) (resid 108 and name HD22) 2.31 0.61 0.61
assign (resid 108 and name  HN) (resid 108 and name   HA) 2.92 0.50 0.50
assign (resid 108 and name  HN) (resid 108 and name  HB1) 3.09 0.84 0.85
assign (resid 108 and name HD22) (resid 108 and name  HB1) 3.35 1.10 1.10
assign (resid 108 and name HD21) (resid 108 and name  HB1) 2.98 0.73 0.73
assign (resid 108 and name HD21) (resid 108 and name  HB2) 2.96 0.71 0.71
assign (resid 108 and name  HN) (resid 109 and name   HN) 2.52 0.72 0.72
assign (resid 108 and name  HN) (resid 109 and name  HB*) 4.24 1.26 1.26
assign (resid 108 and name  HN) (resid 117 and name  HG1) 3.10 0.54 0.54
assign (resid 109 and name  HN) (resid 107 and name   HA) 4.38 0.76 0.77
assign (resid 109 and name  HN) (resid 108 and name   HA) 4.08 1.08 1.08
assign (resid 109 and name  HN) (resid 108 and name  HB2) 4.05 1.55 1.55
assign (resid 109 and name  HN) (resid 109 and name   HA) 2.83 0.58 0.58
assign (resid 109 and name  HN) (resid 109 and name  HB*) 3.98 1.11 1.11
assign (resid 109 and name  HN) (resid 110 and name   HN) 3.60 1.10 1.10
assign (resid 110 and name  HN) (resid 109 and name  HA)  2.48 0.43 0.43
assign (resid 110 and name  HN) (resid 110 and name  HA)  2.92 0.50 0.50
assign (resid 110 and name  HN) (resid 110 and name  HB1) 3.34 0.58 0.59
assign (resid 110 and name  HN) (resid 110 and name  HB2) 2.94 0.52 0.52
assign (resid 110 and name  HN) (resid 111 and name  HN)  2.35 0.41 0.41
assign (resid 110 and name  HN) (resid 112 and name  HN)  3.29 0.58 0.57
assign (resid 111 and name  HN) (resid 111 and name  HA1) 3.30 1.33 1.33
assign (resid 111 and name  HN) (resid 111 and name  HA2) 3.15 1.27 1.27
assign (resid 111 and name  HN) (resid 112 and name  HN)  3.27 1.32 1.32
assign (resid 112 and name  HN) (resid 111 and name  HA1) 3.02 0.53 0.53
assign (resid 112 and name  HN) (resid 111 and name  HA2) 3.04 0.53 0.53
assign (resid 112 and name  HN) (resid 112 and name  HA)  2.92 0.50 0.50
assign (resid 112 and name  HN) (resid 112 and name  HB2) 3.12 0.88 0.88
assign (resid 112 and name  HN) (resid 112 and name  HB1) 3.12 0.88 0.88
assign (resid 113 and name  HN) (resid 102 and name  HZ)  2.90 0.51 0.51
assign (resid 113 and name  HN) (resid 112 and name  HA)  2.34 0.41 0.41
assign (resid 113 and name  HN) (resid 112 and name  HB1) 2.92 0.57 0.58
assign (resid 113 and name  HN) (resid 112 and name  HB2) 2.94 0.56 0.56
assign (resid 113 and name  HN) (resid 113 and name  HA)  2.92 0.50 0.50
assign (resid 113 and name  HN) (resid 113 and name  HB)  2.52 0.44 0.44
assign (resid 113 and name  HN) (resid 113 and name HG12) 3.23 0.98 0.98
assign (resid 113 and name  HN) (resid 113 and name HG2*) 3.52 1.02 1.02
assign (resid 113 and name  HN) (resid 113 and name HG11) 3.24 0.77 0.76
assign (resid 113 and name  HN) (resid 113 and name HD1*) 3.47 1.22 1.22
assign (resid 113 and name  HN) (resid 148 and name  HB1) 3.13 0.55 0.55
assign (resid 113 and name  HN) (resid 149 and name  HN)  2.75 0.48 0.48
assign (resid 113 and name  HN) (resid 149 and name  HB)  2.86 0.50 0.50
assign (resid 113 and name  HN) (resid 150 and name  HA)  3.12 0.54 0.54
assign (resid 114 and name  HN) (resid 113 and name  HA)  2.61 0.81 0.81
assign (resid 114 and name  HN) (resid 113 and name HG2*) 4.00 1.50 1.50
assign (resid 114 and name  HN) (resid 114 and name  HA)  2.92 0.50 0.50
assign (resid 114 and name  HN) (resid 114 and name  HB2) 3.77 1.27 1.27
assign (resid 114 and name  HN) (resid 117 and name  HB1) 3.69 0.65 0.65
assign (resid 114 and name  HN) (resid 117 and name  HG1) 3.79 1.29 1.29
assign (resid 115 and name  HN) (resid 114 and name  HA)  2.62 0.46 0.46
assign (resid 115 and name  HN) (resid 114 and name  HB1) 2.77 0.48 0.48
assign (resid 115 and name  HN) (resid 114 and name  HB2) 2.91 0.66 0.65
assign (resid 115 and name  HN) (resid 115 and name  HA)  2.92 0.50 0.50
assign (resid 115 and name  HN) (resid 115 and name  HB)  2.51 0.44 0.44
assign (resid 115 and name  HN) (resid 115 and name HG11) 3.69 0.72 0.71
assign (resid 115 and name  HN) (resid 115 and name HG2*) 3.17 0.97 0.97
assign (resid 115 and name  HN) (resid 116 and name  HN)  3.05 0.53 0.53
assign (resid 116 and name  HN) (resid 114 and name  HA)  3.52 0.68 0.68
assign (resid 116 and name  HN) (resid 115 and name  HA)  3.41 0.60 0.59
assign (resid 116 and name  HN) (resid 115 and name HG2*) 3.19 0.97 0.97
assign (resid 116 and name  HN) (resid 115 and name HD1*) 3.84 1.16 1.16
assign (resid 116 and name  HN) (resid 115 and name  HB)  3.21 0.56 0.56
assign (resid 116 and name  HN) (resid 116 and name  HA)  2.92 0.50 0.50
assign (resid 116 and name  HN) (resid 116 and name  HG1) 2.98 0.73 0.73
assign (resid 116 and name  HN) (resid 116 and name  HB1) 2.51 0.44 0.44
assign (resid 116 and name  HN) (resid 116 and name  HB2) 3.35 0.58 0.58
assign (resid 116 and name  HN) (resid 117 and name  HN)  2.70 0.47 0.47
assign (resid 116 and name  HN) (resid 118 and name  HN)  3.52 0.69 0.69
assign (resid 117 and name  HN) (resid 113 and name HG2*) 4.45 2.10 2.09
assign (resid 117 and name  HN) (resid 116 and name  HB1) 3.19 1.29 1.29
assign (resid 117 and name  HN) (resid 116 and name  HG1) 3.34 1.54 1.54
assign (resid 117 and name  HN) (resid 116 and name  HB2) 3.89 1.57 1.58
assign (resid 117 and name  HN) (resid 117 and name  HG1) 3.26 1.32 1.32
assign (resid 117 and name  HN) (resid 117 and name  HB1) 3.23 1.31 1.31
assign (resid 117 and name  HN) (resid 118 and name  HN)  3.35 1.36 1.35
assign (resid 118 and name  HN) (resid 113 and name HG2*) 3.23 0.98 0.98
assign (resid 118 and name  HN) (resid 115 and name  HA)  2.92 0.50 0.50
assign (resid 118 and name  HN) (resid 117 and name  HB1) 3.05 0.80 0.81
assign (resid 118 and name  HN) (resid 118 and name   HA) 2.92 0.50 0.50
assign (resid 118 and name  HN) (resid 118 and name  HB2) 2.80 0.49 0.49
assign (resid 118 and name  HN) (resid 119 and name   HN) 3.14 0.55 0.55
assign (resid 118 and name  HN) (resid 121 and name HD1*) 4.08 1.12 1.12
assign (resid 119 and name  HN) (resid 115 and name HG11) 4.06 1.64 1.64
assign (resid 119 and name  HN) (resid 116 and name   HA) 3.54 1.43 1.43
assign (resid 119 and name  HN) (resid 118 and name  HB2) 3.68 1.49 1.50
assign (resid 119 and name  HN) (resid 119 and name  HA1) 2.83 1.15 1.15
assign (resid 119 and name  HN) (resid 119 and name  HA2) 3.02 1.22 1.23
assign (resid 119 and name  HN) (resid 120 and name   HN) 3.46 1.40 1.40
assign (resid 119 and name  HN) (resid 134 and name HG2*) 3.85 1.86 1.85
assign (resid 120 and name  HN) (resid 119 and name  HA2) 3.85 1.56 1.56
assign (resid 120 and name  HN) (resid 119 and name  HA1) 3.35 1.36 1.36
assign (resid 120 and name  HN) (resid 120 and name  HB1) 2.93 1.19 1.19
assign (resid 121 and name  HN) (resid 118 and name   HA) 3.83 1.75 1.75
assign (resid 121 and name  HN) (resid 119 and name  HA2) 4.25 1.93 1.93
assign (resid 121 and name  HN) (resid 120 and name   HA) 2.77 1.26 1.27
assign (resid 121 and name  HN) (resid 120 and name  HG*) 3.85 2.02 2.02
assign (resid 121 and name  HN) (resid 120 and name  HB2) 2.71 1.23 1.24
assign (resid 121 and name  HN) (resid 121 and name   HB) 2.96 1.35 1.35
assign (resid 121 and name  HN) (resid 121 and name HG11) 3.09 1.40 1.41
assign (resid 121 and name  HN) (resid 121 and name HD1*) 4.04 2.11 2.11
assign (resid 121 and name  HN) (resid 122 and name   HN) 3.17 1.44 1.45
assign (resid 122 and name  HN) (resid 119 and name  HA1) 3.58 1.17 1.17
assign (resid 122 and name  HN) (resid 119 and name  HA2) 3.78 0.66 0.66
assign (resid 122 and name  HN) (resid 121 and name HG2*) 3.82 1.08 1.08
assign (resid 122 and name  HN) (resid 122 and name   HA) 2.92 0.50 0.50
assign (resid 122 and name  HN) (resid 122 and name  HB1) 2.80 1.00 1.00
assign (resid 122 and name  HN) (resid 122 and name  HB2) 2.81 1.01 1.00
assign (resid 122 and name  HN) (resid 122 and name HD2*) 3.86 1.09 1.09
assign (resid 122 and name  HN) (resid 123 and name   HN) 2.98 0.73 0.73
assign (resid 123 and name  HN) (resid 120 and name   HA) 2.92 0.50 0.50
assign (resid 123 and name  HN) (resid 122 and name   HA) 3.55 0.62 0.62
assign (resid 123 and name  HN) (resid 122 and name  HB1) 2.98 0.52 0.52
assign (resid 123 and name  HN) (resid 122 and name  HB2) 3.44 0.94 0.94
assign (resid 123 and name  HN) (resid 123 and name   HA) 2.92 0.50 0.50
assign (resid 123 and name  HN) (resid 123 and name  HB*) 3.15 0.97 0.96
assign (resid 123 and name  HN) (resid 123 and name  HG2) 3.32 0.58 0.58
assign (resid 123 and name  HN) (resid 124 and name   HN) 2.81 0.50 0.50
assign (resid 123 and name  HN) (resid 125 and name   HN) 4.11 0.72 0.71
assign (resid 123 and name  HN) (resid 134 and name HD1*) 4.23 1.15 1.16
assign (resid 124 and name  HN) (resid 123 and name   HA) 3.30 0.57 0.58
assign (resid 124 and name  HN) (resid 123 and name  HB*) 3.48 1.02 1.02
assign (resid 124 and name  HN) (resid 124 and name   HA) 2.92 0.50 0.50
assign (resid 124 and name  HN) (resid 124 and name  HB*) 2.95 0.93 0.93
assign (resid 124 and name  HN) (resid 125 and name   HN) 3.04 0.53 0.53
assign (resid 124 and name  HN) (resid 125 and name HG2*) 4.25 1.16 1.16
assign (resid 125 and name  HN) (resid 122 and name   HA) 3.97 0.69 0.70
assign (resid 125 and name  HN) (resid 123 and name   HA) 3.75 0.66 0.66
assign (resid 125 and name  HN) (resid 124 and name   HA) 2.96 0.64 0.64
assign (resid 125 and name  HN) (resid 124 and name  HB*) 3.77 1.07 1.07
assign (resid 125 and name  HN) (resid 125 and name   HA) 2.92 0.50 0.50
assign (resid 125 and name  HN) (resid 125 and name HG2*) 3.45 1.02 1.01
assign (resid 125 and name  HN) (resid 126 and name   HN) 3.05 0.53 0.53
assign (resid 126 and name  HN) (resid 123 and name   HA) 2.52 1.19 1.19
assign (resid 126 and name  HN) (resid 125 and name   HA) 2.77 1.26 1.27
assign (resid 126 and name  HN) (resid 126 and name  HA1) 2.45 1.11 1.11
assign (resid 126 and name  HN) (resid 126 and name  HA2) 2.40 1.09 1.09
assign (resid 126 and name  HN) (resid 127 and name   HN) 2.99 1.36 1.36
assign (resid 127 and name  HN) (resid 125 and name   HA) 3.39 1.02 1.02
assign (resid 127 and name  HN) (resid 126 and name  HA1) 3.25 0.56 0.56
assign (resid 127 and name  HN) (resid 126 and name  HA2) 3.29 0.57 0.58
assign (resid 127 and name  HN) (resid 127 and name   HA) 2.92 0.50 0.50
assign (resid 127 and name  HN) (resid 127 and name  HG1) 3.04 0.79 0.79
assign (resid 127 and name  HN) (resid 127 and name  HB1) 2.73 0.93 0.92
assign (resid 127 and name  HN) (resid 127 and name  HB2) 2.54 0.74 0.75
assign (resid 128 and name  HN) (resid 127 and name   HA) 2.77 1.26 1.27
assign (resid 129 and name  HN) (resid 128 and name   HA) 3.02 0.77 0.77
assign (resid 129 and name  HN) (resid 129 and name   HA) 2.92 0.50 0.50
assign (resid 129 and name  HN) (resid 129 and name   HB) 2.65 0.46 0.46
assign (resid 129 and name  HN) (resid 129 and name HG2*) 3.33 1.18 1.17
assign (resid 130 and name  HN) (resid 129 and name   HA) 1.98 0.34 0.35
assign (resid 130 and name  HN) (resid 129 and name   HB) 3.08 0.68 0.67
assign (resid 130 and name  HN) (resid 129 and name HG2*) 3.25 0.98 0.98
assign (resid 130 and name  HN) (resid 130 and name   HA) 2.92 0.50 0.50
assign (resid 130 and name  HN) (resid 130 and name   HB) 2.89 0.61 0.61
assign (resid 130 and name  HN) (resid 130 and name HG1*) 2.77 0.97 0.97
assign (resid 130 and name  HN) (resid 130 and name HG2*) 3.16 0.97 0.96
assign (resid 130 and name  HN) (resid 133 and name  HB1) 3.02 0.52 0.52
assign (resid 131 and name  HN) (resid 130 and name   HB) 3.00 0.52 0.52
assign (resid 131 and name  HN) (resid 130 and name HG2*) 4.01 1.11 1.12
assign (resid 131 and name  HN) (resid 130 and name   HA) 2.57 0.77 0.77
assign (resid 131 and name  HN) (resid 131 and name   HA) 2.92 0.50 0.50
assign (resid 131 and name  HN) (resid 131 and name  HB2) 2.65 0.85 0.85
assign (resid 131 and name  HN) (resid 131 and name  HB1) 2.66 0.86 0.86
assign (resid 131 and name  HN) (resid 131 and name  HG2) 2.89 1.09 1.08
assign (resid 132 and name  HN) (resid 130 and name HG1*) 3.87 1.09 1.08
assign (resid 132 and name  HN) (resid 130 and name HD1*) 3.79 1.08 1.08
assign (resid 132 and name  HN) (resid 131 and name   HA) 3.54 0.62 0.62
assign (resid 132 and name  HN) (resid 131 and name  HG2) 3.36 0.86 0.86
assign (resid 132 and name  HN) (resid 132 and name   HA) 2.92 0.50 0.50
assign (resid 132 and name  HN) (resid 132 and name  HB*) 3.21 0.98 0.97
assign (resid 132 and name  HN) (resid 133 and name   HN) 2.92 0.67 0.67
assign (resid 133 and name  HN) (resid 130 and name   HN) 3.42 0.60 0.59
assign (resid 133 and name  HN) (resid 130 and name HG1*) 3.21 0.98 0.97
assign (resid 133 and name  HN) (resid 130 and name HD1*) 2.97 0.93 0.93
assign (resid 133 and name  HN) (resid 130 and name   HB) 2.24 0.39 0.39
assign (resid 133 and name  HN) (resid 131 and name   HA) 3.39 0.59 0.59
assign (resid 133 and name  HN) (resid 133 and name   HA) 2.92 0.50 0.50
assign (resid 133 and name  HN) (resid 133 and name  HB1) 2.44 0.64 0.65
assign (resid 133 and name  HN) (resid 133 and name  HB2) 2.58 0.78 0.79
assign (resid 134 and name  HN) (resid 129 and name HG2*) 3.80 1.08 1.08
assign (resid 134 and name  HN) (resid 131 and name   HA) 3.33 0.58 0.58
assign (resid 134 and name  HN) (resid 133 and name   HA) 3.33 0.58 0.58
assign (resid 134 and name  HN) (resid 133 and name  HB1) 2.96 0.71 0.71
assign (resid 134 and name  HN) (resid 133 and name  HB2) 3.29 0.57 0.58
assign (resid 134 and name  HN) (resid 134 and name   HA) 2.92 0.50 0.50
assign (resid 134 and name  HN) (resid 134 and name   HB) 2.57 0.45 0.45
assign (resid 134 and name  HN) (resid 134 and name HG1*) 3.17 0.97 0.97
assign (resid 134 and name  HN) (resid 134 and name HG2*) 3.48 1.02 1.02
assign (resid 134 and name  HN) (resid 134 and name HD1*) 3.29 0.98 0.99
assign (resid 134 and name  HN) (resid 135 and name   HN) 2.66 0.47 0.46
assign (resid 135 and name  HN) (resid 131 and name   HA) 2.92 0.50 0.50
assign (resid 135 and name  HN) (resid 132 and name   HA) 3.44 0.60 0.60
assign (resid 135 and name  HN) (resid 134 and name HG1*) 4.71 1.23 1.24
assign (resid 135 and name  HN) (resid 134 and name   HA) 3.69 0.65 0.65
assign (resid 135 and name  HN) (resid 134 and name   HB) 2.55 0.44 0.45
assign (resid 135 and name  HN) (resid 135 and name   HA) 2.92 0.50 0.50
assign (resid 136 and name  HN) (resid 133 and name   HA) 4.09 0.71 0.71
assign (resid 136 and name  HN) (resid 135 and name   HN) 3.11 0.86 0.86
assign (resid 136 and name  HN) (resid 135 and name  HB1) 2.61 0.81 0.81
assign (resid 136 and name  HN) (resid 135 and name   HA) 3.67 0.65 0.64
assign (resid 136 and name  HN) (resid 135 and name  HB2) 3.05 0.53 0.54
assign (resid 136 and name  HN) (resid 136 and name  HB1) 2.53 0.73 0.73
assign (resid 136 and name  HN) (resid 136 and name  HB2) 2.68 0.88 0.88
assign (resid 136 and name  HN) (resid 136 and name   HA) 2.92 0.50 0.50
assign (resid 136 and name  HN) (resid 137 and name   HN) 3.15 0.55 0.55
assign (resid 136 and name  HN) (resid 139 and name  HG2) 3.42 0.69 0.68
assign (resid 137 and name  HN) (resid 134 and name   HA) 3.40 0.60 0.60
assign (resid 137 and name  HN) (resid 136 and name  HB1) 3.16 0.66 0.65
assign (resid 137 and name  HN) (resid 136 and name  HB2) 3.23 0.73 0.73
assign (resid 137 and name  HN) (resid 136 and name   HA) 3.63 0.63 0.63
assign (resid 137 and name  HN) (resid 137 and name HD1*) 3.77 1.23 1.23
assign (resid 137 and name  HN) (resid 137 and name HD2*) 3.87 1.33 1.33
assign (resid 137 and name  HN) (resid 137 and name  HB1) 2.53 0.73 0.73
assign (resid 137 and name  HN) (resid 137 and name  HB2) 2.46 0.43 0.43
assign (resid 137 and name  HN) (resid 137 and name   HA) 2.92 0.50 0.50
assign (resid 137 and name  HN) (resid 138 and name   HN) 3.05 0.53 0.53
assign (resid 138 and name  HN) (resid 135 and name   HA) 3.23 0.56 0.56
assign (resid 138 and name  HN) (resid 137 and name  HB1) 2.58 0.78 0.79
assign (resid 138 and name  HN) (resid 137 and name  HB2) 2.80 1.00 1.00
assign (resid 138 and name  HN) (resid 137 and name   HG) 3.45 0.60 0.60
assign (resid 138 and name  HN) (resid 138 and name   HA) 2.92 0.50 0.50
assign (resid 138 and name  HN) (resid 138 and name  HG1) 3.87 0.68 0.67
assign (resid 138 and name  HN) (resid 139 and name   HN) 3.03 0.53 0.53
assign (resid 139 and name  HN) (resid 138 and name   HA) 3.30 0.57 0.58
assign (resid 139 and name  HN) (resid 139 and name   HA) 2.92 0.50 0.50
assign (resid 139 and name  HN) (resid 139 and name  HB*) 2.92 0.92 0.93
assign (resid 139 and name  HN) (resid 139 and name  HG1) 2.65 0.85 0.85
assign (resid 139 and name  HN) (resid 139 and name  HG2) 2.77 0.97 0.96
assign (resid 139 and name  HN) (resid 140 and name   HN) 2.85 0.50 0.50
assign (resid 140 and name  HN) (resid 137 and name   HA) 2.72 1.24 1.24
assign (resid 140 and name  HN) (resid 139 and name   HA) 2.77 1.26 1.27
assign (resid 140 and name  HN) (resid 139 and name  HB*) 2.90 1.59 1.58
assign (resid 140 and name  HN) (resid 139 and name  HG2) 3.49 1.59 1.58
assign (resid 140 and name  HN) (resid 139 and name  HD*) 3.95 2.07 2.07
assign (resid 140 and name  HN) (resid 139 and name  HG1) 3.42 1.55 1.55
assign (resid 140 and name  HN) (resid 140 and name  HB1) 2.55 1.16 1.16
assign (resid 140 and name  HN) (resid 140 and name  HB2) 2.79 1.27 1.26
assign (resid 140 and name  HN) (resid 141 and name   HN) 2.75 1.25 1.25
assign (resid 141 and name  HN) (resid 138 and name   HA) 3.10 0.54 0.54
assign (resid 141 and name  HN) (resid 139 and name   HN) 3.75 0.66 0.66
assign (resid 141 and name  HN) (resid 140 and name   HA) 3.00 0.59 0.60
assign (resid 141 and name  HN) (resid 141 and name   HA) 2.92 0.50 0.50
assign (resid 141 and name  HN) (resid 141 and name  HB1) 3.16 0.91 0.91
assign (resid 141 and name  HN) (resid 141 and name  HB2) 2.93 0.68 0.68
assign (resid 141 and name  HN) (resid 142 and name   HN) 2.89 0.64 0.64
assign (resid 142 and name  HN) (resid 138 and name   HA) 3.13 1.33 1.33
assign (resid 142 and name  HN) (resid 139 and name   HA) 2.71 1.23 1.23
assign (resid 142 and name  HN) (resid 140 and name   HN) 2.62 1.23 1.23
assign (resid 142 and name  HN) (resid 141 and name   HA) 2.77 1.26 1.27
assign (resid 142 and name  HN) (resid 141 and name  HB2) 2.84 1.29 1.29
assign (resid 142 and name  HN) (resid 142 and name  HB1) 2.21 1.00 1.01
assign (resid 142 and name  HN) (resid 142 and name  HB2) 2.35 1.07 1.06
assign (resid 143 and name  HN) (resid 143 and name   HA) 2.92 0.50 0.50
assign (resid 143 and name  HN) (resid 143 and name  HG1) 2.44 0.64 0.64
assign (resid 143 and name  HN) (resid 143 and name  HD1) 2.42 0.62 0.62
assign (resid 143 and name  HN) (resid 144 and name   HN) 2.73 0.48 0.48
assign (resid 144 and name  HN) (resid 143 and name   HA) 3.14 0.55 0.55
assign (resid 144 and name HD21) (resid 144 and name  HB2) 3.09 0.84 0.84
assign (resid 144 and name HD21) (resid 144 and name  HB1) 2.96 0.71 0.71
assign (resid 144 and name  HN) (resid 144 and name   HA) 2.92 0.50 0.50
assign (resid 144 and name  HN) (resid 144 and name  HB2) 2.96 0.71 0.71
assign (resid 144 and name HD22) (resid 144 and name  HB1) 3.16 0.55 0.55
assign (resid 144 and name HD22) (resid 144 and name  HB2) 3.41 0.60 0.59
assign (resid 144 and name  HN) (resid 145 and name  HN) 2.63 0.46 0.46
assign (resid 144 and name  HN) (resid 145 and name  HA) 3.59 0.91 0.91
assign (resid 145 and name  HN) (resid 143 and name  HN) 4.21 1.21 1.21
assign (resid 145 and name  HN) (resid 143 and name  HA) 4.69 0.82 0.82
assign (resid 145 and name  HN) (resid 144 and name  HA) 3.88 0.88 0.88
assign (resid 145 and name  HN) (resid 144 and name HB2) 4.28 1.28 1.28
assign (resid 145 and name  HN) (resid 145 and name  HA) 2.83 0.58 0.58
assign (resid 145 and name  HN) (resid 145 and name HB1) 3.63 1.13 1.13
assign (resid 145 and name  HN) (resid 145 and name HB2) 3.53 1.03 1.03
assign (resid 145 and name  HN) (resid 146 and name  HN) 3.54 1.04 1.04
assign (resid 146 and name  HN) (resid 145 and name  HA) 2.56 0.76 0.75
assign (resid 146 and name  HN) (resid 146 and name  HA) 2.92 0.50 0.50
assign (resid 146 and name  HN) (resid 146 and name HB2) 2.90 0.65 0.65
assign (resid 146 and name  HN) (resid 147 and name  HN) 2.37 0.57 0.57
assign (resid 147 and name  HN) (resid 147 and name HA1) 3.33 1.35 1.34
assign (resid 147 and name  HN) (resid 147 and name HA2) 3.16 1.28 1.28
assign (resid 147 and name  HN) (resid 148 and name  HN) 3.26 1.32 1.33
assign (resid 148 and name  HN) (resid 146 and name  HN) 3.49 0.72 0.71
assign (resid 148 and name  HN) (resid 147 and name HA1) 2.88 0.50 0.50
assign (resid 148 and name  HN) (resid 147 and name HA2) 3.00 0.60 0.60
assign (resid 148 and name  HN) (resid 148 and name  HA) 2.92 0.50 0.50
assign (resid 148 and name  HN) (resid 148 and name HB1) 2.92 0.67 0.68
assign (resid 148 and name  HN) (resid 148 and name HB2) 2.88 0.62 0.62
assign (resid 148 and name  HN) (resid 148 and name HG1) 3.17 0.62 0.62
assign (resid 148 and name  HN) (resid 148 and name HG2) 3.54 0.72 0.71
assign (resid 149 and name  HN) (resid 113 and name  HB) 2.70 0.48 0.47
assign (resid 149 and name  HN) (resid 114 and name  HA) 3.19 0.56 0.56
assign (resid 149 and name  HN) (resid 138 and name HE*) 3.75 1.06 1.06
assign (resid 149 and name  HN) (resid 148 and name  HA) 2.27 0.40 0.40
assign (resid 149 and name  HN) (resid 148 and name HB1) 2.66 0.47 0.46
assign (resid 149 and name  HN) (resid 148 and name HB2) 3.07 0.54 0.54
assign (resid 149 and name  HN) (resid 148 and name HG1) 3.14 0.66 0.66
assign (resid 149 and name  HN) (resid 149 and name  HA) 2.92 0.50 0.50
assign (resid 149 and name  HN) (resid 149 and name  HB) 2.50 0.44 0.44
assign (resid 149 and name  HN) (resid 149 and name HG1*) 3.71 1.06 1.06
assign (resid 149 and name  HN) (resid 149 and name HD1*) 3.42 1.01 1.01
assign (resid 150 and name  HN) (resid 149 and name  HN) 4.29 0.75 0.75
assign (resid 150 and name  HN) (resid 149 and name  HA) 2.61 0.81 0.81
assign (resid 150 and name  HN) (resid 149 and name  HB) 3.84 0.67 0.67
assign (resid 150 and name  HN) (resid 149 and name HG2*) 4.32 1.17 1.17
assign (resid 150 and name  HN) (resid 150 and name  HA) 2.92 0.50 0.50
assign (resid 150 and name  HN) (resid 153 and name  HN) 4.13 1.13 1.13
assign (resid 151 and name  HN) (resid 102 and name  HZ) 2.99 0.74 0.74
assign (resid 151 and name  HN) (resid 150 and name  HA) 2.60 0.46 0.46
assign (resid 151 and name  HN) (resid 150 and name HB1) 2.59 0.79 0.79
assign (resid 151 and name  HN) (resid 150 and name HB2) 2.71 0.91 0.91
assign (resid 151 and name  HN) (resid 151 and name HB2) 3.04 0.90 0.91
assign (resid 151 and name  HN) (resid 151 and name  HA) 2.92 0.50 0.50
assign (resid 151 and name  HN) (resid 151 and name HB1) 2.59 0.45 0.45
assign (resid 151 and name  HN) (resid 152 and name  HN) 3.14 0.55 0.55
assign (resid 152 and name  HN) (resid 102 and name  HZ) 3.94 1.26 1.26
assign (resid 152 and name  HN) (resid 150 and name HB1) 3.10 1.30 1.31
assign (resid 152 and name  HN) (resid 150 and name HB2) 3.19 1.39 1.39
assign (resid 152 and name  HN) (resid 151 and name  HA) 3.37 0.59 0.59
assign (resid 152 and name  HN) (resid 151 and name HB2) 3.49 0.61 0.61
assign (resid 152 and name  HN) (resid 152 and name HB1) 3.30 0.57 0.58
assign (resid 152 and name  HN) (resid 152 and name  HA) 2.92 0.50 0.50
assign (resid 152 and name  HN) (resid 153 and name  HN) 2.91 0.51 0.50
assign (resid 152 and name  HN) (resid 154 and name  HN) 3.75 0.66 0.66
assign (resid 153 and name  HN) (resid 152 and name  HA) 3.30 0.58 0.58
assign (resid 153 and name  HN) (resid 152 and name HB1) 2.79 0.49 0.49
assign (resid 153 and name  HN) (resid 152 and name HB2) 2.55 0.44 0.45
assign (resid 153 and name  HN) (resid 153 and name  HA) 2.92 0.50 0.50
assign (resid 153 and name  HN) (resid 154 and name  HN) 2.87 0.50 0.50
assign (resid 154 and name  HN) (resid 149 and name HG2*) 3.19 0.97 0.97
assign (resid 154 and name  HN) (resid 150 and name  HN) 3.50 0.70 0.70
assign (resid 154 and name  HN) (resid 151 and name  HA) 3.06 0.54 0.54
assign (resid 154 and name  HN) (resid 152 and name  HB2) 3.50 1.00 1.00
assign (resid 154 and name  HN) (resid 154 and name  HA) 2.92 0.50 0.50
assign (resid 154 and name  HN) (resid 154 and name  HB1) 2.67 0.47 0.47
assign (resid 154 and name  HN) (resid 154 and name  HB2) 2.59 0.45 0.46
assign (resid 154 and name  HN) (resid 155 and name  HN) 2.81 0.49 0.49
assign (resid 154 and name  HN) (resid 155 and name  HG) 3.59 0.71 0.71
assign (resid 154 and name  HN) (resid 157 and name  HB*) 3.64 1.05 1.05
assign (resid 155 and name  HN) (resid 154 and name  HB1) 3.06 0.54 0.54
assign (resid 155 and name  HN) (resid 154 and name  HB2) 3.40 0.60 0.60
assign (resid 155 and name  HN) (resid 155 and name  HA) 2.92 0.50 0.50
assign (resid 155 and name  HN) (resid 155 and name  HB1) 3.11 0.89 0.89
assign (resid 155 and name  HN) (resid 155 and name  HG) 2.43 0.63 0.63
assign (resid 155 and name  HN) (resid 155 and name  HB2) 2.94 0.69 0.69
assign (resid 155 and name  HN) (resid 155 and name HD2*) 3.64 1.05 1.05
assign (resid 155 and name  HN) (resid 155 and name HD1*) 3.64 1.05 1.05
assign (resid 155 and name  HN) (resid 156 and name  HN) 3.05 0.53 0.54
assign (resid 156 and name  HN) (resid 153 and name  HA) 3.00 0.59 0.60
assign (resid 156 and name  HN) (resid 155 and name  HG) 3.54 0.62 0.62
assign (resid 156 and name  HN) (resid 155 and name  HB2) 2.79 0.99 0.98
assign (resid 156 and name  HN) (resid 155 and name  HB1) 2.91 0.66 0.65
assign (resid 156 and name  HN) (resid 155 and name  HA) 3.35 0.59 0.59
assign (resid 156 and name  HN) (resid 156 and name  HB*) 3.19 0.97 0.97
assign (resid 156 and name  HN) (resid 156 and name  HA) 2.92 0.50 0.50
assign (resid 156 and name  HN) (resid 157 and name  HN) 2.91 0.66 0.66
assign (resid 157 and name  HN) (resid 154 and name  HA) 3.42 0.60 0.59
assign (resid 157 and name  HN) (resid 155 and name  HB1) 4.12 1.08 1.08
assign (resid 157 and name  HN) (resid 156 and name  HA) 3.19 0.56 0.56
assign (resid 157 and name  HN) (resid 156 and name  HB*) 3.42 1.00 1.00
assign (resid 157 and name  HN) (resid 157 and name  HA) 2.92 0.50 0.50
assign (resid 157 and name  HN) (resid 157 and name  HG1) 3.49 0.61 0.61
assign (resid 157 and name  HN) (resid 157 and name  HB*) 3.17 0.96 0.96
assign (resid 157 and name  HN) (resid 157 and name  HG2) 3.53 0.68 0.67
assign (resid 157 and name  HN) (resid 158 and name  HN) 2.79 0.48 0.48
assign (resid 158 and name  HN) (resid 154 and name  HA) 2.92 1.33 1.33
assign (resid 158 and name  HN) (resid 155 and name  HA) 2.93 1.33 1.33
assign (resid 158 and name  HN) (resid 157 and name  HA) 2.77 1.26 1.27
assign (resid 158 and name  HN) (resid 158 and name  HB1) 2.19 1.00 1.00
assign (resid 158 and name  HN) (resid 158 and name  HB2) 2.33 1.06 1.05
assign (resid 158 and name  HN) (resid 159 and name  HN) 2.97 1.35 1.35
assign (resid 160 and name  HN) (resid 158 and name  HN) 3.42 1.58 1.58
assign (resid 160 and name  HN) (resid 158 and name  HB1) 4.07 1.85 1.85
assign (resid 160 and name  HN) (resid 159 and name  HN) 3.60 1.64 1.64
assign (resid 160 and name  HN) (resid 159 and name  HA) 2.77 1.26 1.27
assign (resid 160 and name  HN) (resid 159 and name  HB*) 4.17 2.17 2.17
assign (resid 160 and name  HN) (resid 160 and name  HA1) 2.77 1.26 1.27
assign (resid 160 and name  HN) (resid 160 and name  HA2) 3.03 1.38 1.38
assign (resid 161 and name  HN) (resid 160 and name  HN) 3.12 0.87 0.87
assign (resid 161 and name  HN) (resid 161 and name  HB) 2.50 0.44 0.44
assign (resid 161 and name  HN) (resid 161 and name  HA) 2.92 0.50 0.50
assign (resid 161 and name  HN) (resid 162 and name  HN) 2.95 0.52 0.51
assign (resid 162 and name  HN) (resid 161 and name  HA) 2.75 0.48 0.48
assign (resid 162 and name  HN) (resid 161 and name  HB) 3.98 0.69 0.69
assign (resid 162 and name  HN) (resid 161 and name HG2*) 4.08 1.12 1.12
assign (resid 162 and name HE21) (resid 162 and name HE22) 2.29 0.59 0.58
assign (resid 162 and name  HN) (resid 162 and name  HA) 2.92 0.50 0.50
assign (resid 162 and name  HN) (resid 162 and name  HB1) 3.31 1.06 1.06
assign (resid 162 and name  HN) (resid 162 and name  HB2) 3.08 0.83 0.82
assign (resid 162 and name HE22) (resid 162 and name  HB1) 4.17 0.73 0.73
assign (resid   5 and name   HN) (resid   6 and name   HA) 4.82 2.22 2.22
assign (resid   8 and name   HN) (resid   4 and name   HB) 2.74 0.94 0.94
assign (resid   8 and name   HN) (resid   4 and name   HA) 2.95 0.80 0.80
assign (resid   8 and name   HN) (resid   8 and name  HB*) 2.89 1.07 1.07
assign (resid  11 and name   HN) (resid   9 and name   HA) 2.90 1.10 1.10
assign (resid  16 and name   HN) (resid  15 and name   HA) 3.05 0.73 0.73
assign (resid  16 and name   HN) (resid  16 and name  HG*) 3.52 1.23 1.23
assign (resid  19 and name   HN) (resid  18 and name  HB2) 2.72 0.65 0.65
assign (resid  20 and name   HN) (resid  20 and name   HA) 2.96 0.71 0.71
assign (resid  23 and name   HN) (resid  22 and name  HB1) 3.22 0.77 0.77
assign (resid  23 and name   HN) (resid  23 and name  HB*) 3.23 1.15 1.15
assign (resid  25 and name   HN) (resid  25 and name  HB*) 4.64 2.40 2.41
assign (resid  28 and name   HN) (resid  28 and name  HG2) 4.12 2.32 2.31
assign (resid  32 and name   HN) (resid  32 and name   HA) 2.96 0.71 0.71
assign (resid  36 and name   HN) (resid  34 and name  HA1) 3.17 0.75 0.75
assign (resid  40 and name   HN) (resid  39 and name   HB) 5.27 3.47 3.46
assign (resid  48 and name   HN) (resid  49 and name  HB1) 4.82 2.22 2.22
assign (resid  50 and name   HN) (resid  51 and name  HB1) 3.25 1.45 1.45
assign (resid  51 and name   HN) (resid  51 and name  HG*) 5.56 2.83 2.83
assign (resid  55 and name   HN) (resid  55 and name  HG*) 3.79 1.99 1.99
assign (resid  61 and name   HN) (resid  61 and name HD1*) 3.83 1.30 1.30
assign (resid  62 and name   HN) (resid  62 and name   HB) 2.54 0.61 0.60
assign (resid  66 and name   HN) (resid  65 and name HG1*) 3.52 1.23 1.23
assign (resid  66 and name   HN) (resid  65 and name   HB) 2.35 0.56 0.56
assign (resid  66 and name   HN) (resid  66 and name   HA) 2.96 0.71 0.71
assign (resid  67 and name   HN) (resid  66 and name   HA) 2.67 0.64 0.64
assign (resid  76 and name   HN) (resid  74 and name  HB2) 4.31 1.98 1.99
assign (resid  78 and name   HN) (resid  78 and name   HA) 2.96 0.71 0.71
assign (resid  80 and name   HN) (resid  77 and name   HA) 3.18 0.76 0.76
assign (resid  83 and name   HN) (resid  10 and name  HB*) 3.85 1.31 1.31
assign (resid  84 and name   HN) (resid  84 and name  HD*) 4.04 1.34 1.34
assign (resid  84 and name   HN) (resid  85 and name  HG1) 3.50 0.83 0.83
assign (resid  85 and name   HN) (resid  82 and name   HA) 3.58 0.86 0.86
assign (resid  93 and name   HN) (resid  93 and name  HB1) 2.50 0.60 0.59
assign (resid  96 and name   HN) (resid  95 and name   HA) 2.73 0.93 0.94
assign (resid  98 and name   HN) (resid  98 and name HD1*) 3.81 1.29 1.29
assign (resid 101 and name   HN) (resid 100 and name  HB1) 3.03 1.23 1.23
assign (resid 101 and name   HN) (resid 100 and name  HB2) 3.00 1.20 1.21
assign (resid 101 and name   HN) (resid 101 and name  HB2) 2.97 1.17 1.17
assign (resid 101 and name   HN) (resid 101 and name  HB1) 2.75 0.95 0.96
assign (resid 101 and name   HN) (resid 101 and name   HA) 2.96 0.71 0.71
assign (resid 104 and name   HN) (resid 101 and name   HA) 2.69 0.64 0.64
assign (resid 108 and name   HN) (resid 108 and name  HB2) 2.79 0.67 0.67
assign (resid 110 and name   HN) (resid 106 and name  HB1) 3.31 1.51 1.52
assign (resid 115 and name   HN) (resid 138 and name  HE*) 3.77 1.97 1.98
assign (resid 117 and name   HN) (resid 117 and name   HA) 2.96 0.71 0.71
assign (resid 119 and name   HN) (resid 134 and name HD1*) 6.39 3.21 3.21
assign (resid 120 and name   HN) (resid 117 and name   HA) 4.54 2.09 2.09
assign (resid 120 and name   HN) (resid 120 and name  HB2) 4.12 1.90 1.89
assign (resid 121 and name   HN) (resid 121 and name HG12) 2.92 1.11 1.12
assign (resid 121 and name   HN) (resid 121 and name   HA) 2.96 0.71 0.71
assign (resid 122 and name   HN) (resid 118 and name   HG) 2.75 0.95 0.95
assign (resid 122 and name   HN) (resid 121 and name   HA) 3.06 1.26 1.26
assign (resid 122 and name   HN) (resid 121 and name   HB) 2.71 0.91 0.92
assign (resid 122 and name   HN) (resid 122 and name   HG) 2.59 0.79 0.79
assign (resid 124 and name   HN) (resid 120 and name  HB1) 3.52 0.98 0.98
assign (resid 124 and name   HN) (resid 121 and name   HA) 3.49 0.84 0.83
assign (resid 132 and name   HN) (resid 132 and name  HG1) 3.20 0.77 0.76
assign (resid 133 and name   HN) (resid 132 and name  HB*) 3.09 1.12 1.12
assign (resid 135 and name   HN) (resid 135 and name  HB1) 2.38 0.57 0.57
assign (resid 138 and name   HN) (resid 138 and name  HG2) 5.17 2.38 2.38
assign (resid 140 and name  HN) (resid 140 and name  HA) 2.96 0.71 0.71
assign (resid 141 and name  HN) (resid 139 and name  HA) 5.95 2.74 2.74
assign (resid 142 and name  HN) (resid 141 and name HB1) 6.09 2.80 2.80
assign (resid 143 and name  HN) (resid 142 and name  HA) 2.67 0.64 0.64
assign (resid 150 and name  HN) (resid 150 and name HB2) 3.87 1.96 1.96
assign (resid 152 and name  HN) (resid 151 and name HB1) 2.54 0.74 0.74
assign (resid 157 and name  HN) (resid 153 and name  HA) 4.39 2.02 2.02
assign (resid 158 and name  HN) (resid 158 and name  HA) 2.96 0.71 0.71
assign (resid 159 and name  HN) (resid 156 and name  HA) 5.41 2.49 2.49
assign (resid 161 and name  HN) (resid 160 and name HA1) 3.48 0.98 0.98
assign (resid 161 and name  HN) (resid 161 and name HG1*) 3.29 1.17 1.17
assign (resid 162 and name  HN) (resid 161 and name HG1*) 4.06 1.35 1.35
assign (resid 162 and name  HN) (resid 162 and name HG*) 3.75 1.95 1.95
assign (resid  13 and name  HA) (resid  13 and name HD*) 4.60 2.35 2.34
assign (resid  13 and name HB1) (resid  13 and name HD*) 4.28 2.48 2.47
assign (resid  86 and name HG1) (resid  13 and name HE*) 5.46 2.41 2.41
assign (resid  83 and name HG2*) (resid  13 and name HD*) 5.95 2.91 2.90
assign (resid  22 and name  HA) (resid  22 and name HD*) 3.58 1.10 1.11
assign (resid  19 and name  HA) (resid  22 and name HD*) 4.19 1.22 1.22
assign (resid  22 and name HB1) (resid  22 and name HD*) 3.66 1.86 1.86
assign (resid  22 and name HB2) (resid  22 and name HD*) 3.65 1.85 1.85
assign (resid  18 and name HB2) (resid  22 and name HD*) 4.72 1.32 1.33
assign (resid  14 and name HB2) (resid  22 and name HD*) 4.08 1.19 1.20
assign (resid  82 and name HE*) (resid  22 and name HE*) 5.09 1.79 1.79
assign (resid  19 and name HG12) (resid  22 and name HD*) 4.55 1.29 1.29
assign (resid  14 and name HD2*) (resid  22 and name HE*) 4.50 1.68 1.68
assign (resid  14 and name HD1*) (resid  22 and name HE*) 4.65 1.71 1.71
assign (resid  79 and name HD2*) (resid  22 and name HD*) 5.01 1.78 1.78
assign (resid  22 and name HD*) (resid  22 and name HE*) 3.55 1.49 1.50
assign (resid  27 and name  HA) (resid  26 and name HD*) 4.35 1.40 1.39
assign (resid  23 and name  HA) (resid  26 and name HD*) 4.48 1.43 1.43
assign (resid  75 and name  HA) (resid  26 and name HD*) 4.23 1.37 1.37
assign (resid  78 and name HB1) (resid  26 and name HD*) 4.52 1.44 1.43
assign (resid  26 and name  HA) (resid  26 and name HD*) 3.41 1.18 1.18
assign (resid  26 and name HB*) (resid  26 and name HD*) 3.94 2.14 2.15
assign (resid  27 and name HB1) (resid  26 and name HD*) 4.63 1.46 1.46
assign (resid  75 and name HB1) (resid  26 and name HD*) 4.54 1.44 1.45
assign (resid  37 and name HD1*) (resid  26 and name HD*) 4.43 1.80 1.80
assign (resid  74 and name  HA) (resid  26 and name HE*) 3.89 1.29 1.29
assign (resid  36 and name  HA) (resid  26 and name HE*) 3.81 1.27 1.27
assign (resid  74 and name  HA) (resid  26 and name  HZ) 3.21 0.75 0.75
assign (resid  36 and name  HA) (resid  26 and name  HZ) 2.67 0.92 0.92
assign (resid  35 and name HA1) (resid  26 and name HE*) 4.46 1.43 1.42
assign (resid  75 and name  HA) (resid  26 and name HE*) 3.60 1.23 1.23
assign (resid  78 and name HB1) (resid  26 and name HE*) 3.55 1.21 1.21
assign (resid  75 and name HB1) (resid  26 and name HE*) 4.13 1.34 1.35
assign (resid  75 and name  HA) (resid  26 and name  HZ) 3.48 1.23 1.23
assign (resid  75 and name HB2) (resid  26 and name HE*) 4.12 1.35 1.34
assign (resid  36 and name HB2) (resid  26 and name  HZ) 4.10 0.96 0.96
assign (resid  37 and name HG1*) (resid  26 and name  HZ) 4.30 1.38 1.39
assign (resid  26 and name HD*) (resid  26 and name  HZ) 4.09 1.34 1.34
assign (resid  26 and name HD*) (resid  26 and name HE*) 3.64 1.61 1.62
assign (resid  78 and name HE*) (resid  26 and name HD*) 4.51 1.82 1.82
assign (resid  78 and name HD*) (resid  26 and name HD*) 4.32 1.77 1.78
assign (resid  42 and name  HA) (resid  29 and name HD*) 4.95 2.21 2.21
assign (resid  26 and name  HA) (resid  29 and name HD*) 4.98 2.22 2.22
assign (resid  29 and name HB1) (resid  29 and name HE*) 5.70 2.50 2.50
assign (resid  46 and name HG1) (resid  29 and name HE*) 5.56 2.44 2.45
assign (resid  42 and name  HG) (resid  29 and name HE*) 4.86 2.18 2.17
assign (resid  45 and name  HB) (resid  29 and name HE*) 5.15 2.29 2.29
assign (resid  37 and name HG2*) (resid  29 and name HD*) 6.08 2.95 2.96
assign (resid  42 and name HD1*) (resid  29 and name HE*) 5.32 2.66 2.66
assign (resid  37 and name HD1*) (resid  29 and name  HD*) 5.00 2.54 2.54
assign (resid  45 and name HG1*) (resid  29 and name  HD*) 5.13 2.59 2.59
assign (resid  45 and name HG1*) (resid  29 and name  HE*) 5.57 2.76 2.76
assign (resid  75 and name   HA) (resid  75 and name  HD*) 3.44 1.19 1.19
assign (resid  78 and name  HB1) (resid  75 and name  HD*) 4.31 1.40 1.40
assign (resid  26 and name  HB*) (resid  75 and name  HD*) 4.51 1.83 1.83
assign (resid  75 and name  HB1) (resid  75 and name  HD*) 3.30 1.50 1.50
assign (resid  75 and name  HB2) (resid  75 and name  HD*) 3.33 1.53 1.53
assign (resid  79 and name   HG) (resid  75 and name  HD*) 4.47 1.44 1.44
assign (resid  79 and name HD1*) (resid  75 and name  HD*) 5.20 2.00 2.00
assign (resid  23 and name   HA) (resid  75 and name  HE*) 3.30 1.50 1.50
assign (resid  23 and name   HA) (resid  75 and name   HZ) 3.67 1.17 1.17
assign (resid  19 and name   HA) (resid  75 and name  HE*) 4.71 1.49 1.50
assign (resid  22 and name  HB1) (resid  75 and name  HE*) 4.07 1.35 1.34
assign (resid  22 and name  HB2) (resid  75 and name   HZ) 3.73 1.23 1.23
assign (resid  22 and name  HB1) (resid  75 and name   HZ) 4.12 0.97 0.97
assign (resid  19 and name   HA) (resid  75 and name   HZ) 4.06 0.96 0.96
assign (resid  23 and name  HD1) (resid  75 and name  HE*) 4.29 1.40 1.40
assign (resid  23 and name  HG*) (resid  75 and name  HE*) 4.42 1.81 1.80
assign (resid  79 and name   HG) (resid  75 and name  HE*) 3.69 1.25 1.25
assign (resid  19 and name HG2*) (resid  75 and name   HZ) 3.69 1.26 1.25
assign (resid  79 and name HD1*) (resid  75 and name  HE*) 4.41 1.81 1.81
assign (resid  79 and name HD2*) (resid  75 and name  HE*) 4.38 1.80 1.81
assign (resid  79 and name HD1*) (resid  75 and name   HZ) 4.48 1.44 1.45
assign (resid  75 and name  HD*) (resid  75 and name   HZ) 4.12 1.35 1.36
assign (resid  75 and name  HD*) (resid  75 and name  HE*) 3.46 1.58 1.58
assign (resid  78 and name  HD*) (resid  75 and name  HD*) 4.21 1.76 1.76
assign (resid  22 and name   HA) (resid  78 and name  HE*) 4.37 1.25 1.25
assign (resid  75 and name   HA) (resid  78 and name  HD*) 4.27 1.23 1.22
assign (resid  78 and name  HB1) (resid  78 and name  HD*) 3.34 1.54 1.54
assign (resid  78 and name  HB2) (resid  78 and name  HD*) 3.38 1.58 1.58
assign (resid  26 and name   HA) (resid  78 and name  HD*) 4.05 1.18 1.19
assign (resid  26 and name  HB*) (resid  78 and name  HD*) 5.06 1.78 1.78
assign (resid  22 and name  HB2) (resid  78 and name  HE*) 4.05 1.18 1.19
assign (resid  26 and name  HB*) (resid  78 and name  HE*) 4.94 1.76 1.75
assign (resid  73 and name HG2*) (resid  78 and name  HD*) 4.38 1.65 1.65
assign (resid  79 and name   HG) (resid  78 and name  HD*) 4.55 1.28 1.28
assign (resid  81 and name  HE*) (resid  78 and name  HD*) 4.47 1.67 1.67
assign (resid  78 and name  HD*) (resid  78 and name  HE*) 3.55 1.49 1.49
assign (resid 103 and name   HA) (resid 102 and name  HD*) 4.14 1.27 1.27
assign (resid 151 and name   HA) (resid 102 and name  HD*) 4.27 1.30 1.30
assign (resid 154 and name  HB1) (resid 102 and name  HD*) 4.33 1.31 1.31
assign (resid 102 and name   HA) (resid 102 and name  HD*) 3.53 1.14 1.14
assign (resid 102 and name  HB1) (resid 102 and name  HD*) 3.25 1.45 1.45
assign (resid 102 and name  HB2) (resid 102 and name  HD*) 3.22 1.42 1.42
assign (resid 103 and name  HB*) (resid 102 and name  HD*) 4.87 1.82 1.82
assign (resid 149 and name HG2*) (resid 102 and name  HD*) 5.13 1.88 1.88
assign (resid 113 and name HD1*) (resid 102 and name  HD*) 4.23 1.68 1.69
assign (resid 151 and name  HB1) (resid 102 and name  HD*) 4.64 1.37 1.38
assign (resid 112 and name   HA) (resid 102 and name   HZ) 2.71 0.96 0.96
assign (resid 150 and name   HA) (resid 102 and name   HZ) 2.95 1.15 1.15
assign (resid 111 and name  HA1) (resid 102 and name  HE*) 4.46 1.54 1.54
assign (resid 151 and name   HA) (resid 102 and name  HE*) 3.91 1.22 1.21
assign (resid 154 and name  HB1) (resid 102 and name  HE*) 3.95 1.23 1.23
assign (resid 154 and name  HB2) (resid 102 and name  HE*) 3.73 1.18 1.18
assign (resid 102 and name  HB1) (resid 102 and name  HE*) 4.54 1.35 1.36
assign (resid 151 and name  HB1) (resid 102 and name  HE*) 4.36 1.32 1.32
assign (resid 106 and name  HB1) (resid 102 and name  HE*) 4.37 1.32 1.31
assign (resid 112 and name  HB2) (resid 102 and name   HZ) 3.83 1.17 1.17
assign (resid 150 and name  HB1) (resid 102 and name   HZ) 3.49 0.74 0.74
assign (resid 149 and name HG2*) (resid 102 and name  HE*) 4.38 1.71 1.71
assign (resid 113 and name HG12) (resid 102 and name  HE*) 4.23 1.29 1.29
assign (resid 113 and name HD1*) (resid 102 and name  HE*) 4.33 1.71 1.71
assign (resid 102 and name  HD*) (resid 102 and name   HZ) 3.93 1.23 1.23
assign (resid 102 and name  HD*) (resid 102 and name  HE*) 3.48 1.52 1.52
assign (resid 151 and name  HD*) (resid 102 and name  HE*) 4.79 1.80 1.80
assign (resid 151 and name  HD*) (resid 102 and name  HD*) 4.27 1.69 1.69
assign (resid 154 and name  HD*) (resid 102 and name  HD*) 4.21 1.68 1.68
assign (resid 154 and name  HE*) (resid 102 and name  HD*) 4.54 1.75 1.75
assign (resid 158 and name  HE*) (resid 105 and name   HZ) 4.86 2.18 2.17
assign (resid 121 and name HG2*) (resid 105 and name   HZ) 4.83 2.17 2.17
assign (resid 113 and name HD1*) (resid 105 and name  HD*) 6.46 3.10 3.10
assign (resid 104 and name HG2*) (resid 105 and name  HD*) 5.28 2.65 2.64
assign (resid 105 and name  HD*) (resid 105 and name   HZ) 4.19 1.92 1.92
assign (resid 112 and name   HA) (resid 112 and name  HD*) 3.56 1.07 1.07
assign (resid 150 and name   HA) (resid 112 and name  HD*) 3.54 1.07 1.08
assign (resid 150 and name   HA) (resid 112 and name  HE*) 4.29 1.21 1.21
assign (resid 150 and name  HB2) (resid 112 and name  HD*) 3.92 1.15 1.14
assign (resid 112 and name  HB1) (resid 112 and name  HD*) 3.16 1.36 1.36
assign (resid 112 and name  HB2) (resid 112 and name  HD*) 3.19 1.39 1.39
assign (resid 150 and name  HB2) (resid 112 and name  HE*) 3.97 1.15 1.16
assign (resid 148 and name  HB1) (resid 112 and name  HD*) 3.92 1.15 1.14
assign (resid 148 and name  HB2) (resid 112 and name  HD*) 4.12 1.18 1.18
assign (resid 148 and name  HG2) (resid 112 and name  HD*) 4.33 1.22 1.22
assign (resid 112 and name  HD*) (resid 112 and name  HE*) 3.54 1.48 1.47
assign (resid  99 and name   HA) (resid 151 and name  HD*) 5.10 1.66 1.66
assign (resid 151 and name   HA) (resid 151 and name  HD*) 3.92 1.62 1.62
assign (resid 154 and name  HB1) (resid 151 and name  HD*) 5.29 1.71 1.71
assign (resid 102 and name   HA) (resid 151 and name  HD*) 5.26 1.70 1.70
assign (resid 102 and name  HB1) (resid 151 and name  HD*) 5.10 1.66 1.66
assign (resid 102 and name  HB2) (resid 151 and name  HD*) 4.83 1.59 1.59
assign (resid 151 and name  HB1) (resid 151 and name  HD*) 3.71 1.91 1.92
assign (resid 151 and name  HB2) (resid 151 and name  HD*) 3.83 2.04 2.04
assign (resid  99 and name   HA) (resid 151 and name  HE*) 4.18 1.43 1.43
assign (resid  99 and name   HA) (resid 151 and name   HZ) 3.98 1.48 1.48
assign (resid 102 and name  HB2) (resid 151 and name  HE*) 4.57 1.52 1.53
assign (resid  98 and name  HB2) (resid 151 and name  HE*) 4.55 2.05 2.05
assign (resid  98 and name  HB2) (resid 151 and name   HZ) 4.21 1.21 1.21
assign (resid  99 and name  HB*) (resid 151 and name  HE*) 5.25 2.07 2.08
assign (resid  99 and name  HB*) (resid 151 and name   HZ) 5.02 2.02 2.01
assign (resid 155 and name HD1*) (resid 151 and name  HE*) 4.44 1.87 1.87
assign (resid 151 and name  HD*) (resid 151 and name  HE*) 3.75 1.69 1.69
assign (resid 154 and name  HE*) (resid 151 and name  HD*) 5.66 2.18 2.17
assign (resid  99 and name   HA) (resid 154 and name  HD*) 4.79 1.31 1.32
assign (resid 154 and name   HA) (resid 154 and name  HD*) 3.62 1.38 1.38
assign (resid  98 and name   HA) (resid 154 and name  HE*) 4.38 1.23 1.24
assign (resid 154 and name  HB2) (resid 154 and name  HD*) 3.42 1.62 1.62
assign (resid 102 and name  HB2) (resid 154 and name  HE*) 4.29 1.79 1.79
assign (resid 101 and name  HB2) (resid 154 and name  HE*) 3.77 1.52 1.52
assign (resid 102 and name   HA) (resid 154 and name  HE*) 3.87 1.14 1.13
assign (resid 101 and name  HB1) (resid 154 and name  HE*) 4.24 1.21 1.21
assign (resid  98 and name  HB2) (resid 154 and name  HD*) 4.38 1.23 1.24
assign (resid 157 and name  HE*) (resid 154 and name  HD*) 4.67 1.69 1.70
assign (resid  98 and name  HB1) (resid 154 and name  HE*) 3.90 1.15 1.15
assign (resid 158 and name  HE*) (resid 154 and name   HZ) 4.21 1.71 1.71
assign (resid  98 and name   HG) (resid 154 and name  HE*) 3.83 1.13 1.13
assign (resid 149 and name HG2*) (resid 154 and name  HD*) 4.27 1.62 1.62
assign (resid 113 and name HD1*) (resid 154 and name  HD*) 4.57 1.68 1.68
assign (resid 113 and name HD1*) (resid 154 and name  HE*) 4.91 1.74 1.74
assign (resid 154 and name  HD*) (resid 154 and name  HE*) 3.54 1.48 1.48
```

2. ϕ Restraints

```
!!
!! Phi restraints: minimum deviation = 30 degrees

 !!   6
assign (resid  5 and name c )  (resid  6 and name n )
     (resid  6 and name ca)  (resid  6 and name c ) 1.0 -60.0 10.0 2
 !!   7
assign (resid  6 and name c )  (resid  7 and name n )
     (resid  7 and name ca)  (resid  7 and name c ) 1.0 -60.0 10.0 2
 !!   8
assign (resid  7 and name c )  (resid  8 and name n )
     (resid  8 and name ca)  (resid  8 and name c ) 1.0 -60.0 10.0 2
 !!   10
assign (resid  9 and name c )  (resid 10 and name n )
     (resid 10 and name ca)  (resid 10 and name c ) 1.0 -60.0 10.0 2
 !!   11
assign (resid 10 and name c )  (resid 11 and name n )
     (resid 11 and name ca)  (resid 11 and name c ) 1.0 -58.0 10.0 2
 !!   12
assign (resid 11 and name c )  (resid 12 and name n )
     (resid 12 and name ca)  (resid 12 and name c ) 1.0 -60.0 10.0 2
 !!   13
assign (resid 12 and name c )  (resid 13 and name n )
     (resid 13 and name ca)  (resid 13 and name c ) 1.0 -86.0 20.0 2
 !!   15
assign (resid 14 and name c )  (resid 15 and name n )
     (resid 15 and name ca)  (resid 15 and name c ) 1.0 -64.0 20.0 2
 !!   17
assign (resid 16 and name c )  (resid 17 and name n )
     (resid 17 and name ca)  (resid 17 and name c ) 1.0 -60.0 10.0 2
 !!   18
assign (resid 17 and name c )  (resid 18 and name n )
     (resid 18 and name ca)  (resid 18 and name c ) 1.0 -60.0 10.0 2
 !!   20
assign (resid 19 and name c )  (resid 20 and name n )
     (resid 20 and name ca)  (resid 20 and name c ) 1.0 -60.0 10.0 2
 !!   21
assign (resid 20 and name c )  (resid 21 and name n )
     (resid 21 and name ca)  (resid 21 and name c ) 1.0 -60.0 10.0 2
 !!   22
assign (resid 21 and name c )  (resid 22 and name n )
     (resid 22 and name ca)  (resid 22 and name c ) 1.0 -60.0 10.0 2
 !!   23
assign (resid 22 and name c )  (resid 23 and name n )
     (resid 23 and name ca)  (resid 23 and name c ) 1.0 -60.0 10.0 2
 !!   24
assign (resid 23 and name c )  (resid 24 and name n )
     (resid 24 and name ca)  (resid 24 and name c ) 1.0 -60.0 10.0 2
 !!   25
assign (resid 24 and name c )  (resid 25 and name n )
     (resid 25 and name ca)  (resid 25 and name c ) 1.0 -60.0 10.0 2
 !!   26
assign (resid 25 and name c )  (resid 26 and name n )
     (resid 26 and name ca)  (resid 26 and name c ) 1.0 -60.0 10.0 2
 !!   27
assign (resid 26 and name c )  (resid 27 and name n )
     (resid 27 and name ca)  (resid 27 and name c ) 1.0 -60.0 10.0 2
 !!   30
assign (resid 29 and name c )  (resid 30 and name n )
```

```
     (resid 30 and name ca)  (resid 30 and name c ) 1.0 -62.0 20.0 2
!!  31
assign (resid 30 and name c )  (resid 31 and name n )
     (resid 31 and name ca)  (resid 31 and name c ) 1.0 -60.0 10.0 2
!!  32
assign (resid 31 and name c )  (resid 32 and name n )
     (resid 32 and name ca)  (resid 32 and name c ) 1.0 -94.0 10.0 2
!!  37
assign (resid 36 and name c )  (resid 37 and name n )
     (resid 37 and name ca)  (resid 37 and name c ) 1.0 -91.0 10.0 2
!!  39
assign (resid 38 and name c )  (resid 39 and name n )
     (resid 39 and name ca)  (resid 39 and name c ) 1.0 -60.0 10.0 2
!!  40
assign (resid 39 and name c )  (resid 40 and name n )
     (resid 40 and name ca)  (resid 40 and name c ) 1.0 -60.0 10.0 2
!!  41
assign (resid 40 and name c )  (resid 41 and name n )
     (resid 41 and name ca)  (resid 41 and name c ) 1.0 -60.0 10.0 2
!!  42
assign (resid 41 and name c )  (resid 42 and name n )
     (resid 42 and name ca)  (resid 42 and name c ) 1.0 -60.0 10.0 2
!!  45
assign (resid 44 and name c )  (resid 45 and name n )
     (resid 45 and name ca)  (resid 45 and name c ) 1.0 -60.0 10.0 2
!!  46
assign (resid 45 and name c )  (resid 46 and name n )
     (resid 46 and name ca)  (resid 46 and name c ) 1.0 -63.0 10.0 2
!!  47
assign (resid 46 and name c )  (resid 47 and name n )
     (resid 47 and name ca)  (resid 47 and name c ) 1.0 -60.0 10.0 2
!!  48
assign (resid 47 and name c )  (resid 48 and name n )
     (resid 48 and name ca)  (resid 48 and name c ) 1.0 -82.0 20.0 2
!!  51
assign (resid 50 and name c )  (resid 51 and name n )
     (resid 51 and name ca)  (resid 51 and name c ) 1.0 -99.0 10.0 2
!!  54
assign (resid 53 and name c )  (resid 54 and name n )
     (resid 54 and name ca)  (resid 54 and name c ) 1.0 -85.0 20.0 2
!!  55
assign (resid 54 and name c )  (resid 55 and name n )
     (resid 55 and name ca)  (resid 55 and name c ) 1.0 -60.0 10.0 2
!!  56
assign (resid 55 and name c )  (resid 56 and name n )
     (resid 56 and name ca)  (resid 56 and name c ) 1.0 -60.0 10.0 2
!!  57
assign (resid 56 and name c )  (resid 57 and name n )
     (resid 57 and name ca)  (resid 57 and name c ) 1.0 -60.0 10.0 2
!!  58
assign (resid 57 and name c )  (resid 58 and name n )
     (resid 58 and name ca)  (resid 58 and name c ) 1.0 -60.0 10.0 2
!!  59
assign (resid 58 and name c )  (resid 59 and name n )
     (resid 59 and name ca)  (resid 59 and name c ) 1.0 -60.0 10.0 2
!!  60
assign (resid 59 and name c )  (resid 60 and name n )
     (resid 60 and name ca)  (resid 60 and name c ) 1.0 -60.0 10.0 2
!!  61
assign (resid 60 and name c )  (resid 61 and name n )
     (resid 61 and name ca)  (resid 61 and name c ) 1.0 -60.0 10.0 2
!!  63
```

```
assign (resid  62 and name c )   (resid  63 and name n )
       (resid  63 and name ca)   (resid  63 and name c )  1.0 -60.0 10.0 2
!!   64
assign (resid  63 and name c )   (resid  64 and name n )
       (resid  64 and name ca)   (resid  64 and name c )  1.0 -64.0 10.0 2
!!   65
assign (resid  64 and name c )   (resid  65 and name n )
       (resid  65 and name ca)   (resid  65 and name c )  1.0 -90.0 20.0 2
!!   66
assign (resid  65 and name c )   (resid  66 and name n )
       (resid  66 and name ca)   (resid  66 and name c )  1.0 -60.0 10.0 2
!!   67
assign (resid  66 and name c )   (resid  67 and name n )
       (resid  67 and name ca)   (resid  67 and name c )  1.0 -60.0 10.0 2
!!   68
assign (resid  67 and name c )   (resid  68 and name n )
       (resid  68 and name ca)   (resid  68 and name c )  1.0 -84.0 20.0 2
!!   70
assign (resid  69 and name c )   (resid  70 and name n )
       (resid  70 and name ca)   (resid  70 and name c )  1.0 -76.0 20.0 2
!!   72
assign (resid  71 and name c )   (resid  72 and name n )
       (resid  72 and name ca)   (resid  72 and name c )  1.0 -98.0 10.0 2
!!   73
assign (resid  72 and name c )   (resid  73 and name n )
       (resid  73 and name ca)   (resid  73 and name c )  1.0 -86.0 20.0 2
!!   74
assign (resid  73 and name c )   (resid  74 and name n )
       (resid  74 and name ca)   (resid  74 and name c )  1.0 -83.0 20.0 2
!!   75
assign (resid  74 and name c )   (resid  75 and name n )
       (resid  75 and name ca)   (resid  75 and name c )  1.0 -60.0 10.0 2
!!   76
assign (resid  75 and name c )   (resid  76 and name n )
       (resid  76 and name ca)   (resid  76 and name c )  1.0 -60.0 10.0 2
!!   77
assign (resid  76 and name c )   (resid  77 and name n )
       (resid  77 and name ca)   (resid  77 and name c )  1.0 -60.0 10.0 2
!!   78
assign (resid  77 and name c )   (resid  78 and name n )
       (resid  78 and name ca)   (resid  78 and name c )  1.0 -60.0 10.0 2
!!   79
assign (resid  78 and name c )   (resid  79 and name n )
       (resid  79 and name ca)   (resid  79 and name c )  1.0 -60.0 10.0 2
!!   80
assign (resid  79 and name c )   (resid  80 and name n )
       (resid  80 and name ca)   (resid  80 and name c )  1.0 -60.0 10.0 2
!!   81
assign (resid  80 and name c )   (resid  81 and name n )
       (resid  81 and name ca)   (resid  81 and name c )  1.0 -60.0 10.0 2
!!   82
assign (resid  81 and name c )   (resid  82 and name n )
       (resid  82 and name ca)   (resid  82 and name c )  1.0 -64.0 10.0 2
!!   83
assign (resid  82 and name c )   (resid  83 and name n )
       (resid  83 and name ca)   (resid  83 and name c )  1.0 -60.0 10.0 2
!!   85
assign (resid  84 and name c )   (resid  85 and name n )
       (resid  85 and name ca)   (resid  85 and name c )  1.0 -120.0 20.0 2
!!   86
assign (resid  85 and name c )   (resid  86 and name n )
       (resid  86 and name ca)   (resid  86 and name c )  1.0 -71.0 30.0 2
```

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!!  87
assign (resid  86 and name c )  (resid  87 and name n )
    (resid  87 and name ca)  (resid  87 and name c ) 1.0 -60.0 30.0 2
!!  88
assign (resid  87 and name c )  (resid  88 and name n )
    (resid  88 and name ca)  (resid  88 and name c ) 1.0 -60.0 30.0 2
!!  89
assign (resid  88 and name c )  (resid  89 and name n )
    (resid  89 and name ca)  (resid  89 and name c ) 1.0 -60.0 30.0 2
!!  90
assign (resid  89 and name c )  (resid  90 and name n )
    (resid  90 and name ca)  (resid  90 and name c ) 1.0 -66.0 10.0 2
!!  91
assign (resid  90 and name c )  (resid  91 and name n )
    (resid  91 and name ca)  (resid  91 and name c ) 1.0 -60.0 10.0 2
!!  93
assign (resid  92 and name c )  (resid  93 and name n )
    (resid  93 and name ca)  (resid  93 and name c ) 1.0 -76.0 20.0 2
!!  94
assign (resid  93 and name c )  (resid  94 and name n )
    (resid  94 and name ca)  (resid  94 and name c ) 1.0 -60.0 10.0 2
!!  95
assign (resid  94 and name c )  (resid  95 and name n )
    (resid  95 and name ca)  (resid  95 and name c ) 1.0 -60.0 10.0 2
!!  96
assign (resid  95 and name c )  (resid  96 and name n )
    (resid  96 and name ca)  (resid  96 and name c ) 1.0 -60.0 10.0 2
!!  97
assign (resid  96 and name c )  (resid  97 and name n )
    (resid  97 and name ca)  (resid  97 and name c ) 1.0 -60.0 10.0 2
!!  98
assign (resid  97 and name c )  (resid  98 and name n )
    (resid  98 and name ca)  (resid  98 and name c ) 1.0 -60.0 10.0 2
!!  99
assign (resid  98 and name c )  (resid  99 and name n )
    (resid  99 and name ca)  (resid  99 and name c ) 1.0 -60.0 10.0 2
!!  101
assign (resid  100 and name c )  (resid  101 and name n )
    (resid  101 and name ca)  (resid  101 and name c ) 1.0 -60.0 10.0 2
!!  102
assign (resid  101 and name c )  (resid  102 and name n )
    (resid  102 and name ca)  (resid  102 and name c ) 1.0 -60.0 10.0 2
!!  103
assign (resid  102 and name c )  (resid  103 and name n )
    (resid  103 and name ca)  (resid  103 and name c ) 1.0 -60.0 10.0 2
!!  105
assign (resid  104 and name c )  (resid  105 and name n )
    (resid  105 and name ca)  (resid  105 and name c ) 1.0 -77.0 20.0 2
!!  107
assign (resid  106 and name c )  (resid  107 and name n )
    (resid  107 and name ca)  (resid  107 and name c ) 1.0 -60.0 10.0 2
!!  108
assign (resid  107 and name c )  (resid  108 and name n )
    (resid  108 and name ca)  (resid  108 and name c ) 1.0 -98.0 10.0 2
!!  109
assign (resid  108 and name c )  (resid  109 and name n )
    (resid  109 and name ca)  (resid  109 and name c ) 1.0  60.0 20.0 2
!!  110
assign (resid  109 and name c )  (resid  110 and name n )
    (resid  110 and name ca)  (resid  110 and name c ) 1.0 -110.0 20.0 2
!!  112
assign (resid  111 and name c )  (resid  112 and name n )
```

```
    (resid  112 and name ca)   (resid  112 and name c )  1.0 -120.0 20.0 2
 !!   113
assign (resid  112 and name c )   (resid  113 and name n )
    (resid  113 and name ca)   (resid  113 and name c )  1.0 -91.0 20.0 2
 !!   114
assign (resid  113 and name c )   (resid  114 and name n )
    (resid  114 and name ca)   (resid  114 and name c )  1.0 -92.0 20.0 2
 !!   115
assign (resid  114 and name c )   (resid  115 and name n )
    (resid  115 and name ca)   (resid  115 and name c )  1.0 -60.0 10.0 2
 !!   116
assign (resid  115 and name c )   (resid  116 and name n )
    (resid  116 and name ca)   (resid  116 and name c )  1.0 -74.0 20.0 2
 !!   117
assign (resid  116 and name c )   (resid  117 and name n )
    (resid  117 and name ca)   (resid  117 and name c )  1.0 -55.0 10.0 2
 !!   118
assign (resid  117 and name c )   (resid  118 and name n )
    (resid  118 and name ca)   (resid  118 and name c )  1.0 -60.0 10.0 2
 !!   120
assign (resid  119 and name c )   (resid  120 and name n )
    (resid  120 and name ca)   (resid  120 and name c )  1.0 -60.0 10.0 2
 !!   121
assign (resid  120 and name c )   (resid  121 and name n )
    (resid  121 and name ca)   (resid  121 and name c )  1.0 -58.0 10.0 2
 !!   122
assign (resid  121 and name c )   (resid  122 and name n )
    (resid  122 and name ca)   (resid  122 and name c )  1.0 -60.0 10.0 2
 !!   123
assign (resid  122 and name c )   (resid  123 and name n )
    (resid  123 and name ca)   (resid  123 and name c )  1.0 -60.0 10.0 2
 !!   124
assign (resid  123 and name c )   (resid  124 and name n )
    (resid  124 and name ca)   (resid  124 and name c )  1.0 -64.0 10.0 2
 !!   125
assign (resid  124 and name c )   (resid  125 and name n )
    (resid  125 and name ca)   (resid  125 and name c )  1.0 -84.0 20.0 2
 !!   127
assign (resid  126 and name c )   (resid  127 and name n )
    (resid  127 and name ca)   (resid  127 and name c )  1.0 -79.0 20.0 2
 !!   129
assign (resid  128 and name c )   (resid  129 and name n )
    (resid  129 and name ca)   (resid  129 and name c )  1.0 -93.0 10.0 2
 !!   130
assign (resid  129 and name c )   (resid  130 and name n )
    (resid  130 and name ca)   (resid  130 and name c )  1.0 -96.0 10.0 2
 !!   131
assign (resid  130 and name c )   (resid  131 and name n )
    (resid  131 and name ca)   (resid  131 and name c )  1.0 -60.0 10.0 2
 !!   132
assign (resid  131 and name c )   (resid  132 and name n )
    (resid  132 and name ca)   (resid  132 and name c )  1.0 -60.0 10.0 2
 !!   134
assign (resid  133 and name c )   (resid  134 and name n )
    (resid  134 and name ca)   (resid  134 and name c )  1.0 -64.0 10.0 2
 !!   135
assign (resid  134 and name c )   (resid  135 and name n )
    (resid  135 and name ca)   (resid  135 and name c )  1.0 -60.0 10.0 2
 !!   136
assign (resid  135 and name c )   (resid  136 and name n )
    (resid  136 and name ca)   (resid  136 and name c )  1.0 -60.0 10.0 2
 !!   137
```

```
assign (resid  136 and name c )   (resid  137 and name n )
       (resid  137 and name ca)   (resid  137 and name c )  1.0 -60.0 10.0 2
!!  138
assign (resid  137 and name c )   (resid  138 and name n )
       (resid  138 and name ca)   (resid  138 and name c )  1.0 -60.0 10.0 2
!!  139
assign (resid  138 and name c )   (resid  139 and name n )
       (resid  139 and name ca)   (resid  139 and name c )  1.0 -60.0 10.0 2
!!  141
assign (resid  140 and name c )   (resid  141 and name n )
       (resid  141 and name ca)   (resid  141 and name c )  1.0 -91.0 10.0 2
!!  142
assign (resid  141 and name c )   (resid  142 and name n )
       (resid  142 and name ca)   (resid  142 and name c )  1.0 -77.0 10.0 2
!!  143
assign (resid  142 and name c )   (resid  143 and name n )
       (resid  143 and name ca)   (resid  143 and name c )  1.0 -64.0 10.0 2
!!  144
assign (resid  143 and name c )   (resid  144 and name n )
       (resid  144 and name ca)   (resid  144 and name c )  1.0 -103.0 10.0 2
!!  145
assign (resid  144 and name c )   (resid  145 and name n )
       (resid  145 and name ca)   (resid  145 and name c )  1.0  60.0 20.0 2
!!  146
assign (resid  145 and name c )   (resid  146 and name n )
       (resid  146 and name ca)   (resid  146 and name c )  1.0 -107.0 10.0 2
!!  148
assign (resid  147 and name c )   (resid  148 and name n )
       (resid  148 and name ca)   (resid  148 and name c )  1.0 -95.0 20.0 2
!!  149
assign (resid  148 and name c )   (resid  149 and name n )
       (resid  149 and name ca)   (resid  149 and name c )  1.0 -96.0 20.0 2
!!  150
assign (resid  149 and name c )   (resid  150 and name n )
       (resid  150 and name ca)   (resid  150 and name c )  1.0 -120.0 20.0 2
!!  151
assign (resid  150 and name c )   (resid  151 and name n )
       (resid  151 and name ca)   (resid  151 and name c )  1.0 -60.0 10.0 2
!!  153
assign (resid  152 and name c )   (resid  153 and name n )
       (resid  153 and name ca)   (resid  153 and name c )  1.0 -58.0 10.0 2
!!  154
assign (resid  153 and name c )   (resid  154 and name n )
       (resid  154 and name ca)   (resid  154 and name c )  1.0 -60.0 10.0 2
!!  156
assign (resid  155 and name c )   (resid  156 and name n )
       (resid  156 and name ca)   (resid  156 and name c )  1.0 -60.0 10.0 2
!!  157
assign (resid  156 and name c )   (resid  157 and name n )
       (resid  157 and name ca)   (resid  157 and name c )  1.0 -60.0 10.0 2
!!  159
assign (resid  158 and name c )   (resid  159 and name n )
       (resid  159 and name ca)   (resid  159 and name c )  1.0 -60.0 10.0 2
!!  161
assign (resid  160 and name c )   (resid  161 and name n )
       (resid  161 and name ca)   (resid  161 and name c )  1.0 -88.0 50.0 2
!!  162
assign (resid  161 and name c )   (resid  162 and name n )
       (resid  162 and name ca)   (resid  162 and name c )  1.0 -88.0 50.0 2
```

3. ψ Restraints

```
!!
!! Psi restraints: minimum deviation = 30 degrees

 !!  7
assign (resid  7 and name n )  (resid  7 and name ca)
       (resid  7 and name c )  (resid  8 and name n ) 1.0 -30.0 110.0 2
 !!  8
assign (resid  8 and name n )  (resid  8 and name ca)
       (resid  8 and name c )  (resid  9 and name n ) 1.0 -30.0 110.0 2
 !!  9
assign (resid  9 and name n )  (resid  9 and name ca)
       (resid  9 and name c )  (resid 10 and name n ) 1.0 -30.0 110.0 2
 !! 12
assign (resid 12 and name n )  (resid 12 and name ca)
       (resid 12 and name c )  (resid 13 and name n ) 1.0 -30.0 110.0 2
 !! 16
assign (resid 16 and name n )  (resid 16 and name ca)
       (resid 16 and name c )  (resid 17 and name n ) 1.0 -30.0 110.0 2
 !! 21
assign (resid 21 and name n )  (resid 21 and name ca)
       (resid 21 and name c )  (resid 22 and name n ) 1.0 -30.0 110.0 2
 !! 22
assign (resid 22 and name n )  (resid 22 and name ca)
       (resid 22 and name c )  (resid 23 and name n ) 1.0 -30.0 110.0 2
 !! 23
assign (resid 23 and name n )  (resid 23 and name ca)
       (resid 23 and name c )  (resid 24 and name n ) 1.0 -30.0 110.0 2
 !! 25
assign (resid 25 and name n )  (resid 25 and name ca)
       (resid 25 and name c )  (resid 26 and name n ) 1.0 -30.0 110.0 2
 !! 26
assign (resid 26 and name n )  (resid 26 and name ca)
       (resid 26 and name c )  (resid 27 and name n ) 1.0 -30.0 110.0 2
 !! 27
assign (resid 27 and name n )  (resid 27 and name ca)
       (resid 27 and name c )  (resid 28 and name n ) 1.0 -30.0 110.0 2
 !! 30
assign (resid 30 and name n )  (resid 30 and name ca)
       (resid 30 and name c )  (resid 31 and name n ) 1.0 120.0 100.0 2
 !! 32
assign (resid 32 and name n )  (resid 32 and name ca)
       (resid 32 and name c )  (resid 33 and name n ) 1.0 -30.0 110.0 2
 !! 36
assign (resid 36 and name n )  (resid 36 and name ca)
       (resid 36 and name c )  (resid 37 and name n ) 1.0 120.0 100.0 2
 !! 39
assign (resid 39 and name n )  (resid 39 and name ca)
       (resid 39 and name c )  (resid 40 and name n ) 1.0 -30.0 110.0 2
 !! 42
assign (resid 42 and name n )  (resid 42 and name ca)
       (resid 42 and name c )  (resid 43 and name n ) 1.0 -30.0 110.0 2
 !! 44
assign (resid 44 and name n )  (resid 44 and name ca)
       (resid 44 and name c )  (resid 45 and name n ) 1.0 -30.0 110.0 2
 !! 45
assign (resid 45 and name n )  (resid 45 and name ca)
       (resid 45 and name c )  (resid 46 and name n ) 1.0 -30.0 110.0 2
 !! 46
assign (resid 46 and name n )  (resid 46 and name ca)
```

```
     (resid 46 and name c )  (resid 47 and name n ) 1.0 -30.0 110.0 2
!! 49
assign (resid 49 and name n )  (resid 49 and name ca)
     (resid 49 and name c )  (resid 50 and name n ) 1.0 -30.0 110.0 2
!! 51
assign (resid 51 and name n )  (resid 51 and name ca)
     (resid 51 and name c )  (resid 52 and name n ) 1.0 120.0 100.0 2
!! 53
assign (resid 53 and name n )  (resid 53 and name ca)
     (resid 53 and name c )  (resid 54 and name n ) 1.0 120.0 100.0 2
!! 55
assign (resid 55 and name n )  (resid 55 and name ca)
     (resid 55 and name c )  (resid 56 and name n ) 1.0 -30.0 110.0 2
!! 56
assign (resid 56 and name n )  (resid 56 and name ca)
     (resid 56 and name c )  (resid 57 and name n ) 1.0 -30.0 110.0 2
!! 58
assign (resid 58 and name n )  (resid 58 and name ca)
     (resid 58 and name c )  (resid 59 and name n ) 1.0 -30.0 110.0 2
!! 59
assign (resid 59 and name n )  (resid 59 and name ca)
     (resid 59 and name c )  (resid 60 and name n ) 1.0 -30.0 110.0 2
!! 62
assign (resid 62 and name n )  (resid 62 and name ca)
     (resid 62 and name c )  (resid 63 and name n ) 1.0 -30.0 110.0 2
!! 63
assign (resid 63 and name n )  (resid 63 and name ca)
     (resid 63 and name c )  (resid 64 and name n ) 1.0 -30.0 110.0 2
!! 64
assign (resid 64 and name n )  (resid 64 and name ca)
     (resid 64 and name c )  (resid 65 and name n ) 1.0 -30.0 110.0 2
!! 67
assign (resid 67 and name n )  (resid 67 and name ca)
     (resid 67 and name c )  (resid 68 and name n ) 1.0 -30.0 110.0 2
!! 68
assign (resid 68 and name n )  (resid 68 and name ca)
     (resid 68 and name c )  (resid 69 and name n ) 1.0 -30.0 110.0 2
!! 72
assign (resid 72 and name n )  (resid 72 and name ca)
     (resid 72 and name c )  (resid 73 and name n ) 1.0 120.0 100.0 2
!! 73
assign (resid 73 and name n )  (resid 73 and name ca)
     (resid 73 and name c )  (resid 74 and name n ) 1.0 120.0 100.0 2
!! 74
assign (resid 74 and name n )  (resid 74 and name ca)
     (resid 74 and name c )  (resid 75 and name n ) 1.0 120.0 100.0 2
!! 76
assign (resid 76 and name n )  (resid 76 and name ca)
     (resid 76 and name c )  (resid 77 and name n ) 1.0 -30.0 110.0 2
!! 79
assign (resid 79 and name n )  (resid 79 and name ca)
     (resid 79 and name c )  (resid 80 and name n ) 1.0 -30.0 110.0 2
!! 81
assign (resid 81 and name n )  (resid 81 and name ca)
     (resid 81 and name c )  (resid 82 and name n ) 1.0 -30.0 110.0 2
!! 91
assign (resid 91 and name n )  (resid 91 and name ca)
     (resid 91 and name c )  (resid 92 and name n ) 1.0 -30.0 110.0 2
!! 94
assign (resid 94 and name n )  (resid 94 and name ca)
     (resid 94 and name c )  (resid 95 and name n ) 1.0 -30.0 110.0 2
!! 97
```

```
assign (resid  97 and name n )  (resid  97 and name ca)
    (resid  97 and name c )  (resid  98 and name n ) 1.0 -30.0 110.0 2
!!  98
assign (resid  98 and name n )  (resid  98 and name ca)
    (resid  98 and name c )  (resid  99 and name n ) 1.0 -30.0 110.0 2
!!  99
assign (resid  99 and name n )  (resid  99 and name ca)
    (resid  99 and name c )  (resid 100 and name n ) 1.0 -30.0 110.0 2
!! 101
assign (resid 101 and name n )  (resid 101 and name ca)
    (resid 101 and name c )  (resid 102 and name n ) 1.0 -30.0 110.0 2
!! 103
assign (resid 103 and name n )  (resid 103 and name ca)
    (resid 103 and name c )  (resid 104 and name n ) 1.0 -30.0 110.0 2
!! 104
assign (resid 104 and name n )  (resid 104 and name ca)
    (resid 104 and name c )  (resid 105 and name n ) 1.0 -30.0 110.0 2
!! 106
assign (resid 106 and name n )  (resid 106 and name ca)
    (resid 106 and name c )  (resid 107 and name n ) 1.0 120.0 100.0 2
!! 107
assign (resid 107 and name n )  (resid 107 and name ca)
    (resid 107 and name c )  (resid 108 and name n ) 1.0 -30.0 110.0 2
!! 108
assign (resid 108 and name n )  (resid 108 and name ca)
    (resid 108 and name c )  (resid 109 and name n ) 1.0 -30.0 110.0 2
!! 109
assign (resid 109 and name n )  (resid 109 and name ca)
    (resid 109 and name c )  (resid 110 and name n ) 1.0 120.0 100.0 2
!! 112
assign (resid 112 and name n )  (resid 112 and name ca)
    (resid 112 and name c )  (resid 113 and name n ) 1.0 120.0 100.0 2
!! 114
assign (resid 114 and name n )  (resid 114 and name ca)
    (resid 114 and name c )  (resid 115 and name n ) 1.0 120.0 100.0 2
!! 115
assign (resid 115 and name n )  (resid 115 and name ca)
    (resid 115 and name c )  (resid 116 and name n ) 1.0 -30.0 110.0 2
!! 116
assign (resid 116 and name n )  (resid 116 and name ca)
    (resid 116 and name c )  (resid 117 and name n ) 1.0 -30.0 110.0 2
!! 121
assign (resid 121 and name n )  (resid 121 and name ca)
    (resid 121 and name c )  (resid 122 and name n ) 1.0 -30.0 110.0 2
!! 123
assign (resid 123 and name n )  (resid 123 and name ca)
    (resid 123 and name c )  (resid 124 and name n ) 1.0 -30.0 110.0 2
!! 125
assign (resid 125 and name n )  (resid 125 and name ca)
    (resid 125 and name c )  (resid 126 and name n ) 1.0 -30.0 110.0 2
!! 128
assign (resid 128 and name n )  (resid 128 and name ca)
    (resid 128 and name c )  (resid 129 and name n ) 1.0 -30.0 110.0 2
!! 129
assign (resid 129 and name n )  (resid 129 and name ca)
    (resid 129 and name c )  (resid 130 and name n ) 1.0 120.0 100.0 2
!! 130
assign (resid 130 and name n )  (resid 130 and name ca)
    (resid 130 and name c )  (resid 131 and name n ) 1.0 120.0 100.0 2
!! 131
assign (resid 131 and name n )  (resid 131 and name ca)
    (resid 131 and name c )  (resid 132 and name n ) 1.0 -30.0 110.0 2
```

```
!! 133
assign (resid 133 and name n )  (resid 133 and name ca)
       (resid 133 and name c )  (resid 134 and name n ) 1.0 -30.0 110.0 2
!! 134
assign (resid 134 and name n )  (resid 134 and name ca)
       (resid 134 and name c )  (resid 135 and name n ) 1.0 -30.0 110.0 2
!! 135
assign (resid 135 and name n )  (resid 135 and name ca)
       (resid 135 and name c )  (resid 136 and name n ) 1.0 -30.0 110.0 2
!! 136
assign (resid 136 and name n )  (resid 136 and name ca)
       (resid 136 and name c )  (resid 137 and name n ) 1.0 -30.0 110.0 2
!! 138
assign (resid 138 and name n )  (resid 138 and name ca)
       (resid 138 and name c )  (resid 139 and name n ) 1.0 -30.0 110.0 2
!! 139
assign (resid 139 and name n )  (resid 139 and name ca)
       (resid 139 and name c )  (resid 140 and name n ) 1.0 -30.0 110.0 2
!! 143
assign (resid 143 and name n )  (resid 143 and name ca)
       (resid 143 and name c )  (resid 144 and name n ) 1.0 -30.0 110.0 2
!! 144
assign (resid 144 and name n )  (resid 144 and name ca)
       (resid 144 and name c )  (resid 145 and name n ) 1.0 -30.0 110.0 2
!! 145
assign (resid 145 and name n )  (resid 145 and name ca)
       (resid 145 and name c )  (resid 146 and name n ) 1.0 120.0 100.0 2
!! 148
assign (resid 148 and name n )  (resid 148 and name ca)
       (resid 148 and name c )  (resid 149 and name n ) 1.0 120.0 100.0 2
!! 150
assign (resid 150 and name n )  (resid 150 and name ca)
       (resid 150 and name c )  (resid 151 and name n ) 1.0 120.0 100.0 2
!! 151
assign (resid 151 and name n )  (resid 151 and name ca)
       (resid 151 and name c )  (resid 152 and name n ) 1.0 -30.0 110.0 2
!! 152
assign (resid 152 and name n )  (resid 152 and name ca)
       (resid 152 and name c )  (resid 153 and name n ) 1.0 -30.0 110.0 2
!! 155
assign (resid 155 and name n )  (resid 155 and name ca)
       (resid 155 and name c )  (resid 156 and name n ) 1.0 -30.0 110.0 2
!! 156
assign (resid 156 and name n )  (resid 156 and name ca)
       (resid 156 and name c )  (resid 157 and name n ) 1.0 -30.0 110.0 2
!! 161
assign (resid 161 and name n )  (resid 161 and name ca)
       (resid 161 and name c )  (resid 162 and name n ) 1.0 120.0 100.0 2
```